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# Chemosensation in *Aedes aegypti*: from molecular response profiles to host-seeking behaviour


## Abstract

The female mosquito *Aedes aegypti* is the primary urban vector of various highly infectious neglected tropical diseases. Disease transmission relies heavily on odour-mediated behaviours, including *e.g.*, sugar- and host-seeking, which are crucial during the mosquito life cycle and has dire impacts on vectorial capacity. Olfaction is, one of the most sophisticated sensory systems, and is the sense by which insects locate and discriminate biologically-relevant odorant volatiles. In female mosquitoes, host-seeking is generally not observed before 2-3 days following adult emergence. Of note, males have also been shown to respond to and orient towards host-associated cues. Vector-borne pathogens can alter the behaviour and physiology of their host, consequently increasing the risk of disease transmission. Thus, the modulation of host-seeking depends on multiple factors, including *e.g.*, age, sex, and infection by a pathogen, which is the main focus of this thesis. During my PhD, I have shown that the modulation of the onset of host-seeking coincides with changes in transcript abundance of chemosensory-related genes expressed in the antennae of newly-emerged mosquitoes. Of these genes, one particular odorant receptor, OR117, exhibits a decrease in transcript abundance as female mosquitoes become capable of host-seeking. Later, I have functionally characterised OR117, together with another odorant receptor-of-interest, OR103, which has previously been shown to be more abundant in the human-preferring subspecies of *Ae. aegypti*. Both OR117 and OR03 are tuned to monoterpenes, and their potential role in the transition between sugar- to host-seeking and human avoidance, respectively, is discussed. Furthermore, I have demonstrated that the locomotion and host-seeking is modulated in dengue-infected females in a time-dependent manner, *i.e.* depending on the time post-infection, and coincides with changes in transcript abundance of genes involved in neural signalling pathways. A better understanding of the modulation of odour-mediated behaviours in mosquitoes is crucial for the development of efficient control strategies against disease vector control.



*Keywords*: chemical ecology, chemosensory-related genes, dengue, deorphanization, electrophysiology, heterologous expression, mosquitoes, olfaction



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# Dedication

To the next generations of PhD students… you are not alone.

"All in all it's *just* another brick in the wall"
(Roger Waters)

And to you, reader, for keeping this thesis alive.

"Do not judge me by my success, judge me by how many times I fell down and got back up again"
(Nelson Mandela)

# Contents

# List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. **Anaïs Karine Tallon**, Sharon Rose Hill, Rickard Ignell (2019). Sex and age modulate antennal chemosensory-related genes linked to the onset of host-seeking in the yellow-fever mosquito, *Aedes aegypti*. Scientific Reports, 9 (43), 1-13.

II. **Anaïs Karine Tallon**, Carolyn S. McBride, Jessica Zung, Sharon Rose Hill, Rickard Ignell. Functional characterisation of two monoterpene odorant receptors linked to floral- and host-selection in *Aedes aegypti* (manuscript)

III. **Anaïs Karine Tallon**, Marcelo Gustavo Lorenzo, Luciano Andrade Moreira, Luis Eduardo Martinez Villegas, Sharon Rose Hill, Rickard Ignell (2020). Dengue infection modulates locomotion and host-seeking in *Aedes aegypti*. Plos Neglected Tropical Diseases (accepted)

IV. Crispus Mbaluto, Pascal M. Ayelo, Alexandra G. Duffy, Anna L. Erdei, **Anaïs Karine Tallon**, Siyang Xia, Gabriela Caballero-Vidal, Urban Spitaler, Magdolna O. Szelényi, Gonçalo Duarte, William B. Walker III, Paul G. Becher. Insect Chemical Ecology: Chemical mediated interaction between plant-insect, and insect-insect, and novel applications in agriculture. Arthropod-Plant Interactions (accepted with minor revisions)

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The contribution of Anaïs Karine Tallon to the papers included in this thesis was as follows:

I. Designed the study, performed the experiments. Analysed and interpreted the data. Wrote the manuscript together with co-authors.

II. Designed the study, performed the experiments. Analysed and interpreted the data. Wrote the manuscript together with co-authors.

III. Designed the study, performed the experiments. Analysed and interpreted the data. Wrote the manuscript together with co-authors.

IV. Wrote the section “Insect Disease Vectors” of the review, together with the co-author Siyang Xia.

# List of figures

# Abbreviations

| | |
|---|---|
| ATSB | Attractive toxic sugar bait |
| CHIKV | Chikungunya virus |
| CRISPR | Clustered regularly interspaced short palindromic repeats |
| CSP | Chemosensory proteins |
| DENV | Dengue virus serotype |
| GC-EAD | Gas chromatography and electroantennographic detection |
| GC-SSR | Gas chromatography single sensillum recording |
| GR | Gustatory receptor |
| GPCR | G protein-couple receptor |
| HLA | Human leukocyte antigen |
| iGluRs | Ionotropic glutamate receptors |
| JH | Juvenile hormone |
| IR | Ionotropic receptor |
| LA | Lactic acid |
| NTD | Neglected tropical disease |
| OBP | Odorant binding protein |
| ODE | Odorant degrading protein |
| OR | Odorant receptor |
| Orco | Olfactory receptor co-receptor |
| ORN | Olfactory receptor neuron |
| PBP | Pheromone binding protein |
| PPK | Pickpocket |
| RNA-seq | RNA-sequencing |
| SNMP | Sensory neuron membrane protein |

| | |
|---|---|
| TRP | Transient receptor potential |
| VOC | Volatile organic compound |
| YF | Yellow fever |
| ZIKV | Zika virus |

# 1. Introduction

The humming buzz emitted by mosquitoes has been one of the most annoying sounds on Earth since at least the Jurassic period (145–200 Ma), and it is estimated that a swarming army of at least 100 trillion mosquitoes patrols the globe, annually (Winegard, 2019). Moreover, mosquitoes are suggested to be responsible for killing almost half of the 108 billion humans who have ever populated the earth since our appearance, ca. 200 000 years ago, making these insects the deadliest animals to humans in the world (Kamerow, 2014; Winegard, 2019). Among the total number of formally recognised species of mosquito, a couple of hundred species blood feed on humans (Fang, 2010), and are therefore considered medically important due to their capacity to transmit pathogens (Scott and Takken, 2012) (<200 of 3 578 species currently identified, as in July 2020; http://mosquito-taxonomic-inventory.info/). About half of the world's population is at risk of dengue, currently the most rapidly spreading vector-borne disease in the world, which is mainly transmitted by the mosquito species *Aedes aegypti* (Bhatt et al., 2013; Agha et al., 2017; World Health Organization, 2017b). In mosquitoes, host-seeking is an essential step during the life cycle of females, in which they make use of host cues in search of proteins present in their host blood (Farjana and Tuno, 2013), and is intimately linked to disease transmission (Bowen, 1991; Takken and Knols, 1999). The main objective of my PhD was to explore the modulation of odour-mediated behaviour in *Ae. aegypti*, particularly host-seeking, through behavioural and molecular perspectives, in the context of olfaction in mosquitoes.

The first part of this thesis emphasises on sugar- and host-seeking, two behaviours inevitably linked, even though profoundly different (Edman et al., 1992; Martinez-Ibarra et al., 1997; Foster and Takken, 2004; Roitberg et al., 2010), which have been shown to impact the mosquito

vectorial capacity (Foster, 1995; Stone and Foster, 2013). The role of these two behaviours and the factors influencing their regulation, *e.g.* age, sex, physiological state, environment, and infection by a pathogen, are discussed in detail. Both sugar and host-seeking rely predominantly on olfaction for the detection and integration of ecologically relevant cues (Takken and Knols, 1999; Gouagna et al., 2010; Montell and Zwiebel, 2016; Peach and Gries, 2020). The role of volatiles guiding plant- and vertebrate-host detection and discrimination in mosquitoes and their relative contribution in the context of sugar- and host-seeking is described. Finally, the apparent association between these two behaviours and its potential implications for vector-borne disease control, are further discussed.

Through a highly sophisticated olfactory system, insects have developed the capacity to discriminate among a myriad of biologically-relevant volatiles, simultaneously emitted at different ratios, within a background of other odorants emanating from host and non-host sources (McIver, 1982; Hansson, 2002; Bruce et al., 2005; Su et al., 2012). In the second part of this thesis, I provide the reader with a description of the organisation and role of the peripheral olfactory system in mosquitoes, including the olfactory organs, chemosensory sensilla and olfactory receptor neurons (ORN), and further describe the function of the main chemosensory-related gene families implicated in the detection of odorant signals. While transcriptome analysis has permitted researchers to correlate changes in the expression patterns of these genes with the modulation of odour-mediated behaviours, functional characterization of select receptors is required to inform about the importance of such regulation in gene expression. The third part of this thesis aims to outline the different parameters currently known to be involved in the regulation of the molecular mechanisms underlying the modulation of odour-mediated behaviour in mosquitoes. Overall, a better understanding of the molecular machinery of mosquito olfaction remains necessary, and may greatly contribute to novel control strategies of disease vectors.

## Box 1. *Aedes aegypti*

*Aedes aegypti* is speculated to originate from sub-Saharan Africa, where three subspecies coexist: the two domestic forms *Ae. aegypti aegypti* and *Ae. aegypti queenslandensis*, and the ancestral and zoophilic *Ae. aegypti*

*spp. formusus* (Mattingly, 1957; Nelson, 1986; McBride et al., 2014). It is noteworthy that *Ae. aegypti aegypti* and *Ae. aegypti queenslandensis* were recently shown to be genomically similar and suggested not to be distinct subspecies (Rašić et al., 2016). To avoid confusion for the readers, when the species name *Ae. aegypti* will be used in this thesis, I will exclusively refer to *Ae. aegypti aegypti*. *Aedes aegypti* has likely been exported via shipping during early exploration phases of the old continent (Christophers, Rickard, 1961; Nelson, 1986; Tatem et al., 2006). Despite successful control systems to eradicate this species from the Americas during the 1940s and -50s, most of the countries became re-infested several decades later (Nelson, 1986). Nowadays, this species is widely distributed across tropical and subtropical regions of the world (Lounibos, 2002; Kraemer et al., 2015a), including Europe and North America, where frequent invasions (Porse et al., 2015; Akiner et al., 2016; Ibañez-Justicia et al., 2017), and global occurrence of associated vector-borne diseases (Weaver and Lecuit, 2015; Leta et al., 2018; Brady and Hay, 2019) are reported each year. In addition to the eponym disease, *Ae. aegypti* is also the primary urban vector of other neglected tropical diseases, including dengue fever, chikungunya, Zika (Kyle and Harris, 2008; Bhatt et al., 2013; Fauci and Morens, 2016a; Mayer et al., 2017; Shragai et al., 2017; World Health Organization, 2017b).

Similarly to all Diptera, mosquitoes exhibit complete metamorphosis, and comparably to all mosquito species, *Ae. aegypti* require aquatic habitats for their development (Nelson, 1986; Clements, 1999; Becker et al., 2010). After egg hatching, individuals pass through four larval instars and a pupal stage before emerging into adults (Becker et al., 2010). *Aedes aegypti* adult mosquitoes are diurnal, mostly urban, endophagic (indoor feeding), and endophilic (indoor resting), as they are generally found in areas with high human densities (Nelson, 1986; Kyle and Harris, 2008; Kraemer et al., 2015a). Described as a domestic species, *Ae. aegypti* is found in close association with human dwellings, generally no more than 100 meters away from houses (Nelson, 1986). This species progressively adapted to anthropophily (preference for human hosts), following multiple "domestication" events, defined as the capacity of colonising and adapting to human habitats (Tabachnick, 1991; Brown et al., 2011, 2014). The frequent and highly antropophilic habits of *Ae. aegypti* (Edman et al., 1992; Scott et al., 2000; Scott and Takken, 2012),

and its tendency for taking multiple blood meals in each gonotrophic cycle (Scott et al., 1993, 2000; Scott and Takken, 2012), represent essential factors involved in disease transmission to humans (Padmanabha et al., 2012). Moreover, the tremendous ecological flexibility of *Ae. aegypti* is reflected by its capacity to adapt to its environment, *e.g.* capacity of flying over considerable distances up to several kilometers (Rowley and Graham, 1968), shift sugar feeding from conventional plant sugar sources to human-derived sweet wastes (Dieng et al., 2017), and ability to exploit any type of water reservoirs as breeding habitats, from artificial man-made containers to ground pools (Nelson, 1986). The potential vectorial success of this mosquito species, and the increased risks of disease transmission, which are particularly high in highly-populated urban areas, have made various regions of the world susceptible sites for invasion by *Ae. aegypti* (Leta et al., 2018).

# 2. The implication of olfaction on the vectorial capacity of mosquitoes

## 2.1 Sugar seeking

Mosquitoes feed on sugar derived from flowers, extra-floral nectaries, fruits and plant tissues (Junnila et al., 2010; Müller et al., 2011; Nyasembe et al., 2018), as well as on plant-derived and homopteran-produced honeydew (Takken and Knols, 1999; Gary and Foster, 2004; Stone and Foster, 2013; Peach et al., 2019a; Barredo and DeGennaro, 2020). Moreover, anthropophilic species, such as *Ae. aegypti*, have been demonstrated to switch from classical sugar diets to human-derived sweet wastes present in their environment (Dieng et al., 2017). As such, plant sugar is a prevalent source of nutrients for adult mosquitoes of both sexes, as it is the only nutrient consumed by males and the most common one for females, besides vertebrate blood (Jaenson and Ameneshewa, 1991; Foster, 1995; Takken and Knols, 1999; Impoinvil et al., 2004). Sugar feeding allows for the synthesis and storage of glycogen and triglyceride reserves (Handel, 1965), and provides energy for somatic functions, *e.g*. flight, survival, and reproduction, which affect the mosquito vectorial capacity (Foster, 1995; Stone and Foster, 2013). Yet, there is a lot of conflicting literature regarding whether sugar feeding is essential (Nayar and Sauerman, 1971; Yuval et al., 1994; Briegel et al., 2001), or facultative in mosquitoes (Mccrae et al., 1976; Edman et al., 1992; Spencer et al., 2005; Olson et al., 2020).

### 2.1.1 Relevant life-history traits affected by sugar feeding

In mosquitoes, the energy for sustained flight is generally not derived from the triglycerides synthetized after the absorption of sugar (Haegel, 1965; Nayar and Van Handel, 1971), but rather from the sugars themselves (Nayar

and Van Handel, 1971; Service, 1977). Moreover, as no increase in glycogen occurs after resting (Nayar and Van Handel, 1971), this suggests that the ingested sugars are likely the main fuel source for flight (Magnarelli, 1978; Martinez-Ibarra et al., 1997). However, when all sugar reserves are depleted, glycogen and triglycerides in sugar-fed mosquitoes can be used as the flight substrate (Nayar and Van Handel, 1971; Service, 1977). Starved female mosquitoes have been shown to fly to exhaustion, and can resume vigorous flight only after a sugar meal (Nayar and Van Handel, 1971). Similarly in males, flight, which is involved in mating, *i.e*. swarming behaviour, requires a considerable amount of energy derived from sugar feeding (Yuval et al., 1994). Interestingly, it should be noted that since flight activation and range are essential parameters in disease transmission, sugar feeding can strongly influence mosquito vectorial capacity (Briegel et al., 2001). For instance, restricted flight capacities in mosquitoes were found to be linked to high prevalence of malaria and increased risks of superinfections as the vector encounters and bites the same hosts repeatedly (Clarke et al., 2002).

Sugar reserves that are not directly used for flight energy, are converted into glycogen and triglyceride reserves (Bidlingmayer and Hem, 1973), which can extend the lifespan of a mosquito by days or weeks (de Meillon et al., 1967; Nayar and Van Handel, 1971; Briegel et al., 2001). However, in general, mosquitoes can live for up to only a few days on their energy reserves from a single sugar meal (Nayar and Van Handel, 1971; Vaidyanathan et al., 2008). Sugar-deprived females of both *Ae. aegypti* and *Aedes albopictus* die almost twice as fast as sugar-fed females (Hancock and Foster, 1993; Upshur et al., 2019). Sugar meals are associated with greater energetic values compared to blood meals alone (Nayar and Sauerman, 1971; Day et al., 1994; Gary and Foster, 2001; Nyasembe et al., 2020). However, sugar consumption following an infected blood meal may enhance the pathogen infection and transmission, as shown for malaria-infected mosquitoes (Kelly, 1996). Moreover, both the survival rates of *Ae. aegypti* in response to high temperatures (Gary et al., 2009; Upshur et al., 2019), and overwintering diapause in *Culex* mosquitoes have been shown to be affected by sugar feeding (Bowen, 1992). Thus, sugar feeding modulates mosquito survival, and consequently impact their vectorial capacity (Nyasembe et al., 2015).

Sugar feeding has also been shown to be necessary to stimulate the production of the first egg clutch in anautogenous mosquitoes (Barlow, 1955; O'Meara, 1985). Under laboratory conditions, female mosquitoes offered blood plus sugar produce more eggs per gonotrophic cycle, and are able to continue laying eggs for a longer period of time following a blood meal, than those provided with blood alone or fed on water

only (de Meillon et al., 1967; Harrington et al., 2001; Mostowy and Foster, 2004). Moreover, sugar feeding does not only influence the stimulation of oviposition, but also the rates of egg maturation and ovarian development, in which *e.g.* sugar-deprived *Ae. aegypti* and *Aedes communis* females fail to complete their gonotrophic cycle following a single blood meal compared to those that have been fed with sucrose (Klowden, 1986; Andersson, 1992). It is noteworthy that sugar-deprived gravid *Ae. aegypti* females exhibit a consistently strong attraction to honey, unlike gravid females that had access to sugar, suggesting that orientation to sugar sources in gravid females mainly depends on energy reserve status (Hancock and Foster, 1993). Interestingly, female fecundity can be impaired as a result of reduced insemination rates by sugar-deprived males (Stone et al., 2009), which is in line with field observations of reduced mating performance in male mosquitoes that cannot obtain sugar at frequent intervals (Gary et al., 2009). Overall, the presence of sugar has a strong influence on the aforementioned parameters, and sugar reserves may affect the population dynamics (Stone et al., 2009), and vectorial capacity of mosquitoes (Nasci, 1986; Yuval et al., 1994; Scott and Takken, 2012).

### 2.1.2 Environmental factors regulating sugar seeking

Mosquito sugar feeding has been reported several times in the literature, yet it has not been studied as intensively as other behaviours, *e.g.* blood feeding and host-seeking, and its importance remains comparatively unknown. Observations of mosquitoes feeding on flowers in nature are relatively rare and arduous to interpret as sugar probing does not automatically lead to sugar ingestion (Stone and Foster, 2013). Moreover, despite the development of chemical tests, notably Van Handel's anthrone test for fructose (Van Handel, 1985), the detection of sugar meals remains challenging as absorption and metabolism of sugar can be fast-occurring process (Handel, 1965; Smith and Kurtz, 1995). In addition, sugar feeding can be influenced by various extrinsic factors (Haegel, 1955; Bidlingmayer and Hem, 1973; Service, 1977; Morris, 1984; Andersson, 1990; Foster, 1995; Martinez-Ibarra et al., 1997; Gary and Foster, 2006; Olson et al., 2020). For instance, mosquitoes exhibit distinct cycles in sugar feeding which are strongly subject to a circadian rhythm (Morris, 1984; Foster, 1995; Gary and Foster, 2006). While many *Culicinae* mosquitoes species feed on plants at noon and in the late afternoon (Yee and Foster, 1992; Canyon et al., 1999a), anopheline mosquitoes almost exclusively feed after sunset (Mccrae et al., 1976; Gary and Foster, 2006). Moreover, a sexual dimorphism in the diel activity patterns of sugar seeking has been described in *Cx. tarsalis*, in which females

increase sugar feeding activity in the early morning while males ingest sugar at night after swarming at dusk (Reisen et al., 1986). The modulation of sugar feeding is therefore highly dependent on daily and diel activity patterns.

Sugar seeking is strongly influenced by the temporal availability of sugar sources due to seasonal fluctuations in temperature and humidity (Haegel, 1955; Bidlingmayer and Hem, 1973; Service, 1977; Andersson, 1990; Olson et al., 2020). It is worth noticing that the variation in the plant blooming leads to changes in the availability in sugar resources, and that this is hypothesized to strongly influence mosquito population dynamics (Peach and Gries, 2020). Moreover, variation in sugar concentrations and contents, as well as plant nectar protein contents which vary extensively between seasons, may play a significant role in the modulation of mosquito sugar seeking (Manda et al., 2007a; Nyasembe et al., 2012), and host-plant selection (de Meillon et al., 1967; Nayar and Van Handel, 1971; Briegel et al., 2001; Vrzal et al., 2010; Naziri et al., 2016). Circadian control of sugar feeding has also been proposed to correspond to temporal adaptations to correlate with the floral species and their peak of nectar production (Grimstad and Defoliart, 1975; Magnarelli, 1979; Rund et al., 2011). As temperature increases, sugar-fed *Ae. aegypti* females exhibit higher activity rates and levels of carbohydrates, suggesting a higher sugar consumption and likely reflecting higher flower visit rates (Upshur et al., 2019). Similarly, during the dry season, *Anopheles sergentii* mosquitoes have been shown to shift plant preferences and mainly tissue-feed on succulent species, *i.e.* plants more resistant to dry season conditions (Junnila et al., 2010). Moreover, *Ae. aegypti* mosquitoes collected in Thailand present higher levels of fructose during the dry season which is associated with low–dengue transmission rates, compared with the rainy and high–dengue transmission season (Spencer et al., 2005). Thus, the availability of plant hosts in their local environment, strongly influence sugar feeding and is a crucial determinant of mosquito population dynamics and vectorial capacity.

Fluctuations in mosquito sugar feeding reflect the spatial heterogeneity in sugar availability and distribution of local vegetation (Haegel, 1955; Bidlingmayer and Hem, 1973; Martinez-Ibarra et al., 1997; Gary and Foster, 2006). For instance, the frequency of sugar feeding is higher in sugar-rich sites (Gu et al., 2011), while the frequency of plant tissue feeding, representing a poor quality sugar source, is higher in sugar-poor habitats (Müller and Schlein, 2005). Notably, changes in sugar availability, *i.e.* spatial heterogeneity, may have dramatic effects on mosquito population sex ratio, as male insemination potential which relies on flight activity, survival and mating, heavily depends on their ability to acquire a sugar meal (Stone and Foster, 2013). Moreover, mosquito population dynamics and the

potential for pathogen transmission have been demonstrated to be regulated mainly by the availability of sugar sources in their local environment (Gu et al., 2011; Ebrahimi et al., 2018). As an example, the number of *An. sergentii* females capable of transmitting malaria, was 4 times greater in sugar-rich sites than in sugar-poor sites, and their vectorial capacity was estimated to be more than 250-fold higher in sugar-rich sites (Gu et al., 2011). These studies highlight the importance of mosquito-plant interactions and the potential of targeting sugar-feeding mosquitoes for the development of novel control methods.

Variation in mosquito sugar feeding behaviour has also been suggested to be driven by the presence/absence of vertebrate hosts in their local environment (Yee and Foster, 1992; Ma and Roitberg, 2008; Stone and Foster, 2013). How the regulation of sugar feeding in mosquitoes is affected by the absence of blood-hosts, in the presence of suitable sugar sources is discussed in the subsection 2.1.4 of this thesis.

### 2.1.3 Onset of sugar seeking and tendency to sugar feed

In mosquitoes, sugar seeking varies according to sex and age, as well as throughout the gonotrophic cycle (Haegel, 1955; Nayar and Sauerman, 1975; Magnarelli, 1978). Adult mosquitoes are not ready for mating immediately after emergence, as males require approximately 24 h to complete hypopygial rotation and initiate swarming (Service, 1977; Smith and Gadawski, 1994). Females develop their host-seeking behaviour within 2-5 days (Morris, 1984; Tallon et al., 2019), although, depending on host availability, females may have to wait a few more days before to take their first blood meal (Stone et al., 2011). Thus, sugar seeking is particularly vital for newly-emerged mosquitoes as plant-derived carbohydrates represent a source of energy for daily activities (see subsection 2.1.1). Field observations have demonstrated that males tend to sugar feed more often and on a more regular basis throughout their adult life cycle compared to females (Gary and Foster, 2006; Stone and Foster, 2013; Olson et al., 2020). In contrast, female nectar feeding takes place primarily soon after adult emergence (Smith and Gadawski, 1994; Martinez-Ibarra et al., 1997), and then declines with age (Smith and Gadawski, 1994; Canyon et al., 1999a; Foster and Takken, 2004). Sugar seeking is therefore dependent on age in both male and female mosquitoes.

Among mosquito species there is a continuum of female dependence for sugar (Steffan and Evenhuis, 1981; Edman et al., 1992; Beier, 1996; Collins and Blackwell, 2000; Spencer et al., 2005). At one extreme, adult female *Toxorhynchites spp.* mosquitoes are non-

haematophagous, and feed exclusively on plant sugars (Steffan and Evenhuis, 1981; Collins and Blackwell, 2000), while at the other extreme, in highly anthropophilic species, sugar feeding has been reported to be restricted and comparatively rare (Edman et al., 1992; Beier, 1996; Spencer et al., 2005). In contrast, zoophilic females tend to feed as frequently on sugar as on blood (Stone and Foster, 2013). In most female anthropophilic mosquito species, sugar feeding occurs more often than blood feeding following adult emergence (Stone et al., 2011), *e.g.* female *Ae. cantans* strongly prefer sugar over blood or rarely bite until after several weeks of sugar feeding (Service, 1977). Although sugar feeding can remain frequent beyond the first gonotrophic cycle and throughout the adult life (Reisen et al., 1986; Andersson, 1990; Martinez-Ibarra et al., 1997), a period of complete refractivity has been shown to occur following a blood meal (Nasci and Edman, 1984; Vargo and Foster, 1984; Christ et al., 2017). Sugar seeking generally resumes following oviposition and prior to the next blood feeding (Hancock and Foster, 1993; Stone and Foster, 2013), *e.g. An. gambiae* females have been shown to wait up to two days following a blood meal before sugar feeding again (Gary and Foster, 2006). In contrast, *Ae. aegypti* females can resume sugar seeking 24 h after a blood meal (Christ et al., 2017). Combined, the expression of sugar seeking in mosquitoes appears to be dependent on sex, age and physiological state, and are important parameters to consider for the elaboration of efficient sugar-based trapping systems.

### 2.1.4 The dynamic interplay between sugar- and host-seeking

The initiation of host-seeking in newly-emerged females has been shown to coincide with the accumulation of lipid reserves acquired during sugar feeding, corresponding to the period of time between adult emergence and a blood meal (Renshaw et al., 1995). Both laboratory and field approaches have shown that mosquito sugar- and host-seeking, although profoundly different behaviours, are intimately linked. For instance, culicine female mosquitoes with daily access to blood and oviposition sites were found to feed on sugar more infrequently and later at night (Briegel and Kaiser, 1973). Moreover, diel frequency of nectar seeking was correlated with host-seeking activity (Andersson, 1990; Yee and Foster, 1992), as well as daily access to blood (Gary and Foster, 2006). For instance, in the presence of blood-host stimuli, female *Anopheles quadrimaculatus, Cx. quinquefasciatus,* and *Ae. triseriatus* exhibit an earlier evening peak of sugar-feeding rhythms compared with females in the absence of host stimuli (Yee and Foster, 1992). While nectar feeding frequencies of host-seeking *culicinae* mosquitoes are

similar to their non-host seeking counterparts (Reisen et al., 1986; Bowen et al., 1994), the proportion of sugar intake by female mosquitoes was found to become negligible when the availability in vertebrate hosts increased (Yee and Foster, 1992; Ma and Roitberg, 2008; Stone and Foster, 2013). In the presence of dense human populations, *Ae. aegypti* females rarely feed on sugar, and exhibit high frequencies of multiple blood meals (Edman et al., 1992; Martinez-Ibarra et al., 1997). This low frequency of sugar feeding in nature is likely not related to the absence or low abundance of nectar sources, but rather the non-utilisation of plant sugar as the main source of energy, consequently resulting in an increased vectorial capacity (Edman et al., 1992).

The absence of sugar sources does not only hamper females with small energy reserves, but can also influence host-seeking and blood feeding, *ergo* their vectorial capacity (Walker and Edman, 1985; Nasci, 1991; Andersson, 1992; Roitberg et al., 2010). Interestingly, the modulation of host biting by sugar feeding is likely not restricted to highly anthropophilic species (Braks et al., 2006). Sugar-deprived *Ae. communis* and *An. gambiae* females are less eager to blood feed and continue to respond to host stimuli following a blood meal, compared to females kept on sugar (Andersson, 1992; Roitberg et al., 2010). Similarly, sugar-deprived *Aedes triseriatus* and *Ae. aegypti* females were reported to be less persistent at biting a defensive host, which has been suggested to increase the risk of multiple blood meals on different hosts (Walker and Edman, 1985; Nasci, 1991), *e.g.* sugar-deprived females *An. gambiae* (Straif and Beier, 1996; Gary and Foster, 2001) and *Ae. aegypti* (Canyon et al., 1999a) exhibit increased blood feeding activities. Thus, sugar feeding has strong impacts on host-seeking and blood feeding behaviours, which highlights the implication of sugar seeking on the vectorial capacity of mosquitoes.

Stronger and faster responses to the vertebrate host have been observed in *An. gambiae* female mosquitoes that have received a sugar meal (Foster and Takken, 2004). In contrast, the likelihood of approaching hosts increases in gravid sugar-deprived *Ae. aegypti* females compared to those fed on sucrose, suggesting a decreased post-blood meal host avoidance likely due to the necessity to take a second blood meal as a result of lower energy reserves (Klowden, 1986). Interestingly, the nutritional state of males also affects female host-seeking, as starved males contain and transfer less protein to females during mating, resulting in a reduced inhibition of host-seeking behaviour by the gravid females (Fernandez and Klowden, 1995). In conclusion, the apparent association between sugar- and host-seeking has considerable implications for vector-borne disease control, as the availability of sugar may reduce the likelihood of approaching hosts and the incidence

of multiple blood meals by female mosquitoes. In elevated human densities, the presence of plants was suggested to reduce the incidence of multiple blood feeding by female *Ae. aegypti* mosquitoes (Martinez-Ibarra et al., 1997), suggesting that higher availability of sugar sources could help reducing arbovirus prevalence in these areas.

## 2.2 Chemical ecology of sugar-seeking

The selection of suitable host plants is crucial for the life history of mosquitoes. It is important to note that during their lifespan, mosquitoes face a large spectrum of stimuli emitted from various plant sources. Even though sugar seeking involves the integration of olfactory (Gouagna et al., 2010), gustatory (Kessler et al., 2015), and visual cues (Bernáth et al., 2016; Peach et al., 2019c), this chapter emphasises the odour-mediated associations between specific vector species and plant hosts (Peach and Gries, 2020). Although a better understanding of the chemical communication that underpins mosquito-plant interactions may provide good insights regarding the development of plant-based mosquito controls, the role of olfactory cues governing plant host choice in mosquitoes and the relative contribution of plant volatiles in the context of sugar seeking remain largely unknown.

### 2.2.1 Host plant selection

In insects, host plant selection refers to the ability to discriminate among host plants and obtain nutrients from fluids or secretions from only a small subgroup of species among the plethora of alternatives that may be present in their local habitat (Stone and Foster, 2013). Various techniques have emerged over the last decades to investigate sugar feeding in mosquitoes, from the notable cold- and hot-anthrone tests developed by Van Handel to determine the presence and quantity of individual sugar meals (Van Handel, 1985), to recent molecular DNA-based approaches used in the identification of plant species foraged upon (Junnila et al., 2010; Nyasembe et al., 2018). In the field, mosquitoes have been shown to be highly selective in their choice of plant hosts (Impoinvil et al., 2004; Manda et al., 2007b; Müller et al., 2011; Samson et al., 2013). For instance, *Ae. albopictus* was reported to exhibit high preference for certain ornamental flowers and wildflowers (Müller et al., 2011), while *An. gambiae* preferred *Parthenium hysterophorus* as host plants (Nikbakhtzadeh et al., 2014; Nyasembe et al., 2015). Under laboratory conditions, the flowering plant *Lantana camara* appears to be the most preferred plant species for *Ae. aegypti* (Wondwosen

and Dawit et al. unpublished data), unlike *Ae. albopictus* which was shown to not feed on this particular species (Upshur *et al.* personal communication). The aforementioned techniques have allowed for the identification of differences in host plant selection among mosquito species.

Various plant species have been shown to be more suitable at increasing mosquito fitness than others (Manda et al., 2007b; Nyasembe et al., 2015; Yu et al., 2016). For instance, *Ae. aegypti*, *Cx. pipiens*, and *Ae. albopictus* with the highest glycogen reserves were collected from local plants exhibiting high nectar contents, suggesting that mosquitoes can discriminate among beneficial plants, and thus actively seek rewarding nutrient sources (Samson et al., 2013; Chen and Kearney, 2015). The increased fitness resulting from feeding on these plants is linked to the mosquito host plant preferences (Manda et al., 2007a; Nyasembe et al., 2012; Yu et al., 2016). For instance, longevity and fecundity were enhanced in *An. gambiae* females that had fed on their preferred plant species, compared to those that had fed on the least preferred plant species (Manda et al., 2007a; Nyasembe et al., 2012). Similarly *Cx. pipiens* survival was found to correspond to its nutritional regime preferences (Yu et al., 2016). These studies reveal the importance and potential to target specific vegetation in the context of disease vector control, *e.g.* to optimise the trapping efficiency of sugar baits based on plant species preferred by mosquitoes, or modify the local plant community to reflect the non-preferred plants of the local mosquitoes.

In insects, the olfactory-mediated attraction to plants can be modulated by associative and non-associative learning, which can be defined as the capacity to tailor behavioural choices to local needs according to past experience (Mccall and Kelly, 2002; Lutz et al., 2017). While olfactory learning of floral signals has been well documented in insect pollinators (Wright and Schiestl, 2009; Riffell, 2011), there is presently little information regarding the ability of mosquitoes in associative learning, as most work until now has focused on host seeking (Mccall and Kelly, 2002; Lutz et al., 2017; Vinauger, 2019). That said, unlike naïve mosquitoes, both conditioned male and female *Cx. quinquefasciatus* are more responsive to mixtures and single compounds, when they were trained with an association of plant-produced volatiles with sugar (Jhumur et al., 2006; Tomberlin et al., 2006). Consequently, learning ability is expected to allow mosquitoes to narrow their search of a sugar source and greatly improve the foraging efficiency, which could be particularly advantageous in highly competitive sugar environments. A better understanding of the ability of mosquitoes to locate and discriminate sugar sources in nature remains therefore essential.

### 2.2.2 Odour-based host plant discrimination

During their lifespan, mosquitoes face a myriad of odorant stimuli emitted from host and non-host plant sources, revealing the importance of odour-based host plant discrimination. Plants emit a wide array of volatile organic compounds (VOCs) mainly rich in terpenes, benzenes, and aldehydes which can be detected by mosquitoes (Healy and Jepson, 1988; Nikbakhtzadeh et al., 2014; Nyasembe and Torto, 2014) (Dekker et al., 2011)., *e.g.*(E)- linalool oxide is as an attractant for *Cx pipiens* (Jhumur et al., 2008), and the monoterpenes, ocimene, camphor and eucalyptol, are detected by *Ae. aegypti* (Tallon *et al.* unpublished data)(Dekker et al., 2011). It should also be noted that some of these plant hosts can emit metabolites which influence pathogen-vector interactions (Nyasembe et al., 2015; Hien et al., 2016). For instance, parthenin, a sesquiterpene lactone emitted from *P. hysterophorus*, is responsible for impairing *Plasmodium falciparum* development in mosquitoes (Balaich et al., 2016). Moreover, *An. gambiae* infected with malaria was described to probe more on sugar odours and sources from its most preferred plant species (Nyasembe et al., 2014).

Not only are mosquitoes more attracted towards certain plant species, they also respond differently to the volatiles emitted by these plants (Nyasembe et al., 2012). For instance, *An. gambiae* females were found to respond more strongly to volatiles that are particularly abundant in the emissions from the preferred nectar source (Nyasembe et al., 2012). Differences in odour detection of specific compound classes among mosquito species also appear to reflect those emitted by their respective natural host plants, *e.g.* benzenoids for *Ae. aegypti*, aldehydes for *Ae. mcintoshi*, and sesquiterpenes and alkenes for *An. gambiae* (Nyasembe et al., 2018). Aside from the detection of unique VOCs from their respective host plants, these three different mosquito species were found to consistently respond to the two monoterpenes β-myrcene and (*E*)-β-ocimene present in all the host plants, suggesting that certain compounds may serve as signature cues in plant location (Nyasembe et al., 2018).

In nature, plant volatiles are emitted at various ratios and rates which vary among and within plant species (Gols et al., 2011). Host plant discrimination by insects has been found to rely on both the composition of plant emissions as well as the emission rates (Nyasembe et al., 2012; Hao et al., 2013). Techniques such as combined gas chromatography (GC) and electroantennographic detection (EAD) have permitted researchers to record mosquito responses to the natural ratios of volatiles present in different host plant headspace profiles. For instance, host plant selection by *Ae. aegypti* has been shown to be linked to volatile emission rates of various floral extracts

(Upshur *et al.* personal communication), and both *Ae. albopictus* and *An. gambiae* are attracted to bioactive compounds emitted by their host plants in low amounts, while avoidance of these compounds is induced at higher concentrations (Nyasembe et al., 2012; Hao et al., 2013) Odour sources with moderate release rates may signal the presence of an attractive hosts, while sources releasing high amounts of plant volatiles inform mosquitoes of a non-preferred host or that they have reached the host (Nyasembe et al., 2012).

In nature, plant volatiles are frequently presented to insects as complex mixtures of several hundred compounds (Nikbakhtzadeh et al., 2014), and single bioactive compounds are generally required to be in the form of an odour blend in order to convey reliable information regarding the characteristics of the sugar source (Natale et al., 2003; Riffell et al., 2009). Although single compounds occasionally are sufficient to elicit a specific behaviour (Menger et al., 2014), the role of volatile compounds as required synergists have become further apparent in mosquitoes (Geier et al., 1999; Nyasembe et al., 2012; Mathew et al., 2013; Wondwosen et al., 2016, 2017). Specific mixtures of plant volatiles can be more attractive to sugar-seeking mosquitoes than individual compounds (Peach et al., 2019b). Moreover, *An. gambiae* mosquitoes have been shown to be differentially attracted towards different synthetic floral blends (Nyasembe et al., 2012). These studies support the hypothesis that mosquitoes rely on specific blends of volatile compounds for the host plant discrimination.

Finally, some of these plant hosts can emit volatile secondary metabolites which influence pathogen-vector interactions (Nyasembe et al., 2015; Hien et al., 2016). For instance, parthenin, a sesquiterpene lactone emitted from *P. hysterophorus*, is responsible for impairing *Plasmodium falciparum* development in mosquitoes (Balaich et al., 2016). Moreover, *An. gambiae* infected with malaria was described to probe more on sugar odours and sources from its most preferred plant species (Nyasembe et al., 2014). Thus, a better knowledge of plant-mosquito interactions may lead to a more efficient use of semiochemicals as a control tool to help reducing the risk of disease transmission.

### 2.2.3 Control of vector populations: emphasis on sugar bait stations

Plant-based attractive lures have the potential advantage of not targeting a specific subgroup of mosquitoes only, as sugar seeking is a prevalent source of nutrients in mosquitoes (Jaenson and Ameneshewa, 1991; Foster, 1995; Takken and Knols, 1999; Impoinvil et al., 2004). The attractive toxic sugar baits (ATSB) which are particularly simple and inexpensive, represent a

powerful tool for mosquito control (Müller et al., 2010b). These "attract and kill" systems require the association of insecticides mixed with sugar, but do not rely on fermentation-derived $CO_2$ to attract mosquitoes, and were reported to significantly reduce mosquito populations during several weeks after application (Müller et al., 2010b; Revay et al., 2014; Junnila et al., 2015). Interestingly, ATSB catching success has been demonstrated in both arid and non-arid environments (Müller et al., 2010b; Qualls et al., 2014), and indoor use has been suggested as an efficient supplementary method to mosquito insecticide-treated nets against *Anopheles arabiensis, An. gambiae sensu stricto*. and *Culex quinquefasciatus* in Tanzania (Stewart et al., 2013). Overall, malaria vectorial capacity was shown to dramatically reduce in areas where ATSB field trials were applied regardless of the availability in sugar resources (Beier et al., 2012). Similarly, the use of ATSB has been predicted to be particularly effective against exophilic mosquito species which would tend to be less affected by host-seeking-oriented methods (Marshall et al., 2013). Thus, the use of ATSB represents a promising control strategy against mosquitoes.

Overall, ATSB applied to flowering vegetation were shown to be better at reducing mosquito populations than ATSB presented in bait stations (Revay et al., 2014), and the addition of bioactive volatile compounds to ATSB was found to lead to higher mortality rates (Junnila et al., 2015; Scott-Fiorenzano et al., 2017), minimising the impacts on non-target species populations (Khallaayoune et al., 2013; Qualls et al., 2014; Fiorenzano et al., 2017). However, when the ATSB is applied to vegetation, non-target organisms are expected to suffer important losses, therefore the use of ATSB presented in a bait station is suggested to be more appropriate in sub-tropical environments where sugar meals are readily available (Revay et al., 2014). Natural sugar sources may compete with the applied ATSB solutions (Beier et al., 2012). Lures made from synthetic volatile compounds used by mosquitoes to locate their preferred plant hosts in nature, are essential to include in the ATSB in order to increase its performance, as sugars are not overly attractive on their own (Müller et al., 2011; Fiorenzano et al., 2017). For instance, the level of attraction of both female and male mosquitoes was reported to be higher on sugar traps baited with attractant stimulants, *e.g.* guava, honey melon or flowering plants (Müller and Schlein, 2006; Müller et al., 2010a). The addition of host plant volatiles therefore greatly enhances the efficiency of ATSBs.

Consequently, a deeper knowledge regarding plant host selectivity in mosquitoes and the use of ATSBs implemented on preferred plant hosts and using volatiles as attractants, unlike random selection of plant species associated with insecticides, will likely offer promising opportunities

in the context of arbovirus surveillance and control, by increasing attraction and target specificity.

## 2.3 Host-seeking

Host-seeking is a crucial step during the mosquito life cycle corresponding to the sequence of behavioural events preceding blood feeding in mosquitoes, which ultimately results in their interaction with vertebrate hosts. This behaviour is intimately linked to disease transmission, and relies heavily on the ability of females to locate a human host and successfully blood feed (Bowen, 1991; Takken and Knols, 1999). With some exceptions, *i.e.* the obligatory nectar-feeding mosquito *Tx. amboinensis* (Corbett, 1967), the feeding behaviour of most adult anautogenous females is characterised by ingestion of host blood which provides a protein source necessary for vitellogenesis (Lehane, 2008).

Anautogenous female mosquitoes are feeding specialists that have developed distinct feeding appendages enabling them to blood-feed (Jové et al., 2020). Females make use of host odors in the search for protein-rich blood (Farjana and Tuno, 2013). Although male mosquitoes do not blood feed, males of some species, do respond and orient towards host odours; a behaviour linked to swarming and mate location (Hartberg, 1971; Cabrera and Jaffe, 2007; Hapairai et al., 2013; da Silva Paixão et al., 2015; Tallon et al., 2019). In female mosquitoes, host-seeking is generally not observed before two to three days following adult emergence (Davis, 1984; Foster and Takken, 2004; Tallon et al., 2019) and is inhibited following a blood meal (Klowden and Briegel, 1994; Farjana and Tuno, 2013). Thus, mosquito host-seeking depends on age, sex and physiological state (Bohbot et al., 2013a; Omondi et al., 2019; Tallon et al., 2019). Although divergence in mosquito host selection between anthropophilic and zoophilic species is unlikely to have been a result of stochastic events, changes in host selection were demonstrated to occur among species, depending on various extrinsic factors, *e.g.* genetic and environmental (Gouck, 1972; McBride et al., 2014). In addition, pathogens can also be responsible for alteration of mosquito host-seeking, increasing the probability of disease transmission (Molyneux and Jefferies, 1986; Weaver et al., 1988; Moore, 1993; Hurd, 2003; Lefevre

et al., 2007; Lefèvre and Thomas, 2008; Colpitts et al., 2011; Bonizzoni et al., 2012; Cator et al., 2012). A better understanding of host-seeking is essential in the development of novel strategies against mosquitoes.

### 2.3.1 Onset of female host-seeking and importance of blood feeding

Following adult emergence, female mosquitoes gradually develop their behavioural and physiological competence to seek and feed on human blood (Davis, 1984; Foster and Takken, 2004). In *e.g., Ae. aegypti* and *Aedes atropalpus*, host-seeking behaviour is not observed prior to two to three days following adult emergence (Davis, 1984; Tallon et al., 2019). Similarly, 1 day old female *An. gambiae*, unlike 4 and 6 days-old, do not respond to human host cues (Takken et al., 1998; Omondi et al., 2015a, 2019). The accumulation of energetic reserves during the larval stages contributes to the initiation of host seeking in adult female mosquitoes (Terzian and Stahler, 1949). For instance, larvae of *Ae. aegypti* reared on a suboptimal diet have a lower tendency to engage in host-seeking once emerging as an adult, compared to larvae reared on an optimal diet (Nasci, 1991). Similarly, even though the temporal patterns of onset of host-seeking are similar in small and large female *An. gambiae*, host responsiveness was lower in small females (Takken et al., 1998), which present a higher probability of multiple feeding (Mitchell and Millian, 1981; Takken et al., 1998). Of note, changes in the olfactory system, *i.e.* expression of chemosensory-related genes and the sensitivity of the olfactory receptor neurons (ORNs), were shown to coincide with the onset of host-seeking (Omondi et al., 2015a, 2019; Tallon et al., 2019), which is discussed in more detailed in the following sections. Thus, in both male and female mosquitoes, the onset of host-seeking is modulated during sexual maturation.

### 2.3.2 Regulation of host-seeking during the first gonotrophic cycle

A period of refractiveness to host-seeking is observed following a blood meal in anautogenous female mosquitoes. Although host-seeking is generally restored after oviposition (Klowden and Lea, 1979b; Klowden, 1981; Bowen et al., 1994), the duration of the inhibition of host-seeking may vary substantially depending on the amount of blood imbided and in between species (Klowden and Briegel, 1994; Farjana and Tuno, 2013). For instance,

*An. gambiae* females have been shown to be rapidly refractory to host cues after blood feeding to repletion, for a period of time of approximately two to three days (Jones and Gubbins, 1978; Takken et al., 2001). Discrepancies among these studies are likely related to the quality of the blood meal, *i.e.* the amount of blood ingested. Moreover, the capacity of taking multiple blood meals was controlled in these studies, and might also account for the observed variation (Klowden and Lea, 1978).

The host-seeking inhibition is caused by two separate endogenous mechanisms; to wit, distention of the gut following blood ingestion (Klowden and Lea, 1979a), and the release of hormonal factors later during egg development (Klowden and Lea, 1979b; Klowden et al., 1987). The initial host-seeking inhibition caused by the abdominal distention following blood ingestion is controlled by stretch mechanosensory receptors in the gut after blood ingestion rather than by blood constituents (Klowden and Lea, 1979a), and last as long as the distended state, *i.e.* until the blood meal has been digested (Brown et al., 1994). In contrast, the long-term humoral inhibition is observed following a successfully triggered vitellogenesis (Klowden and Lea, 1979b; Klowden et al., 1987). It has previously been demonstrated that the injection of hemolymph from blood-fed females (Brown et al., 1994), haemolymph-borne neuropeptides (Klowden, 1981; Klowden and Blackmer, 1987; Brown et al., 1994), or synthetic peptides activating neuropeptide Y-like receptors (Liesch et al., 2013; Christ et al., 2017; Duvall et al., 2019) were involved in the suppression of host attraction in non-blood-fed females.

Host-seeking inhibition has been shown to also depend on the body size of females (Farjana and Tuno, 2013), as well as the initial size of the blood meal and the nutritional state (Klowden and Briegel, 1994). Size-dependent suppression of host-seeking has been observed in *Ae. aegypti* in which small females tend to take small or partial blood meals that are insufficient to terminate host-seeking, suggesting that small mosquitoes have more contacts with hosts, compared to large females (Mitchell and Millian, 1981; Farjana and Tuno, 2013). The necessity of supplemental feeding may result in higher propensities to take multiple blood meals (Walker and Edman, 1985; Nasci, 1991; Straif and Beier, 1996; Canyon et al., 1999a; Gary and Foster, 2001). Multiple feeding within a single gonotrophic cycle is a regular part of mosquito biology (Koella et al., 1998; Scott and Takken, 2012), which can be influenced by the density of conspecifics (Basáñez et

al., 2007) and human hosts (Michael et al., 2001), as well as age and body size (Xue et al., 1995; Scott et al., 2000; Farjana and Tuno, 2013). The distention-induced inhibition was shown to depend on the volume of blood ingested, suggesting a certain threshold for the size of the blood meal required to initiate the inhibition of host-seeking (Klowden and Lea, 1978, 1979a; Klowden, 1987). Thus, modulation of host-seeking inhibition may increase the propensity of female mosquitoes to take multiple blood meals, consequently increasing the risk of disease transmission.

### 2.3.3 Host-seeking in male mosquitoes, an often neglected sex

Similarly to other insect disease vectors, *e.g.* fleas (Mears et al., 2002), ticks (Carroll, 2002), triatomines (Bodin et al., 2009), and tsetse flies (Colvin and Gibson, 1992), mosquito host-seeking is not exclusive to females, as males of some species can be found in close association with vertebrate hosts and respond to their odour (Hartberg, 1971; Cabrera and Jaffe, 2007; da Silva Paixão et al., 2015; Tallon et al., 2019). Although males have a crucial role in the maintenance of mosquito populations, host-seeking in male mosquitoes has been under-investigated.

Not only do male mosquitoes respond to host-associated cues, but they can also orient towards hosts. For example, in French Polynesia, a higher proportion of *Ae. aegypti* males were collected in human-baited traps, compared to females (Hapairai et al., 2013). Host-seeking in *Ae. aegypti* males is age dependent, as in females, with newly-emerged males not responding to human odours, while males older than three days post-emergence exhibit an increased flight activity towards host odour (Cabrera and Jaffe, 2007; da Silva Paixão et al., 2015; Tallon et al., 2019). Moreover, mated males are attracted by host odour, while unmated males display an increased flight activity in response to their exposure to the presence of vertebrate hosts (Cabrera and Jaffe, 2007; da Silva Paixão et al., 2015). Field observations have revealed that swarms of *Ae. albopictus* males generally occur around the feet and ankles of human hosts, which also corresponds to the preferred landing sites of females (Shirai et al., 2002). Thus, host-seeking in male *Aedes* mosquitoes have been linked to swarming and mate location (Cabrera and Jaffe, 2007), and mating, *i.e.* successful female insemination, which commonly takes place in close association with the host (Hartberg, 1971). In contrast, *Anopheles* and *Culex* male mosquitoes engage in swarms

even in the absence of vertebrate hosts, primarily using sound and visual cues (GIBSON, 1985; Charlwood et al., 2003; Facchinelli et al., 2015). While males are an often-neglected sex in the study of mosquito host-seeking behaviour, further investigation is required to understand the ecological role of male mosquitoes and their implication in vectorial capacity of mosquito populations.

### 2.3.4 Divergence of host selection

In mosquitoes, host selection depends on various intrinsic and extrinsic factors (Gouck, 1972; McBride et al., 2014). Dietary specialism has been predicted to have evolved from trade-offs between obtaining a greater fitness by feeding on various resources and a higher energy gain when only a limited variety of resources is available, while generalism is advantageous when little difference is found between the resources available (Egas et al., 2004). In mosquitoes, in order to maximize their feeding success, specialism has been hypothesized to have evolved in environments where the availability of suitable vertebrate hosts is high, while the frequency of generalist behaviours are predicted to increase in regions where encounters with suitable hosts are less probable (Kelly and Thompson, 2000). Differences in mosquito host selection is unlikely random, as when faced with the choice between human and non-human animal hosts, anthropophilic species show a decided preference for humans, while zoophilic species prefer to feed on non-human animals (Gouck, 1972; McBride et al., 2014). The mosquito *Ae. aegypti* provides a good example of non-random host selection and specialization in biting on humans. The domestic subspecies *Ae. aegypti aegypti*, which still coexists with its ancestral zoophilic subspecies *Ae. aegypti formosus* along the coast of Kenya, has evolved a strong preference for human hosts (McBride et al., 2014). In addition, genetic variation in host choice has been documented in natural populations of *An. gambiae*, which exhibit shifts in feeding preferences between cows and human hosts after relatively few generations (Gillies, 1964). Similarly, cross-breeding experiments between the zoophilic mosquito species *Aedes simpsoni* and the anthropophilic *Ae. aegypti*, resulted in hybrids with intermediate host preferences, suggesting that the determinant factor for host choice likely depends on the genotype of the male parent (Mukwaya, 1977). Combined, these studies have contributed

to a better knowledge regarding the mechanisms and factors regulating host selection in mosquitoes.

As specialization on one specific type of diet limits mosquitoes to a finite subset of resources while many are available, blood feeding habits of some species have been shown to be more or less flexible than others (Bruce-Chwatt et al., 1966; Burkot et al., 1988; Lothrop and Reisen, 2001; Sousa et al., 2001). For instance, *An. farauti* female have been shown to blood feed on various host types, both human and non-human animals (Bruce-Chwatt et al., 1966; Burkot et al., 1988). Differences in host preferences can also be influenced by geography. For example, the malaria vector *An. gambiae* is highly anthropophilic in mainland Africa (Costantini et al., 1999), while exophilic populations on the island of Sao Tomé were reported to predominantly feed on dogs (Sousa et al., 2001). Seasonal variations also play an important role in the shifts in feeding pattern, from mammal to avian hosts, as observed in *Culex univittatus* during the rainy season in Western Kenya (Chandler et al., 1977), and in *Culex tarsalis* and *Culex nigripalpus* in spring in North America (Kilpatrick et al., 2006). Transformation in landscape and land-use activities are also speculated to be responsible for changes in human preferences in mosquitoes, in which a ten year-study has shown that forest clearing and land drainage due to the intensification of rural agriculture, have contributed to an increase of the human biting rate by *An. fluvialitis* by 2800 % in northern regions of India (Bruce-Chwatt et al., 1966). Similarly, the proportion of blood meals taken on humans by *An. darlingi* was reported to be 278 times higher in deforested regions of the Amazon basin, compared to forested areas (Vittor et al., 2006). Further investigation of changes in environmental conditions underlying the extensive variation in host preference observed among mosquito species remains necessary.

In mosquitoes, human-biting rates and human blood indexes have been reported to be strongly influenced by host availability (Burkot et al., 1989). Studies in western Kenya revealed that the human blood index of *Anopheles* mosquitoes caught inside houses was more prevalent when compared to outdoor environments (Beier et al., 1990; Petrarca et al., 1991). Similarly, human biting rates by *Ae. aegypti* were shown to increase with the availability of human hosts (Canyon et al., 1999b). Host availability not only influences blood feeding behaviour, but may also impact host selection (Lyimo and Ferguson, 2009). For instance, the opportunistic mosquito *An.*

*arabiensis* exhibited a high degree of blood feeding on humans in the absence of cattle (Mwangangi et al., 2003). The demonstrated non-random host selection has been attributed to variation in the nutritional value of the obtained blood, and the energetic costs linked to the digestion of blood of different host types (Lyimo and Ferguson, 2009). For instance, *Cx. tarsalis* females blood-fed on their preferred hosts exhibit faster rates of digestion and lay more eggs, compared to females fed on their non-preferred hosts (Downe and Archer, 1975). An improved understanding of the divergence in mosquito host preferences may help to refine estimates of the risk of disease transmission, and develop efficient intervention methods, *e.g.* zooprophylaxis (Bøgh et al., 2001; Saul, 2003; Mahande et al., 2007).

### 2.3.5 Modulation of host-seeking by pathogen infection

In mosquitoes, the presence of vector-borne pathogens has been suggested to induce alterations in the behaviour and physiology of their hosts, increasing the probability of disease transmission (Molyneux and Jefferies, 1986; Weaver et al., 1988; Moore, 1993; Hurd, 2003; Lefevre et al., 2007; Lefèvre and Thomas, 2008; Colpitts et al., 2011; Bonizzoni et al., 2012; Cator et al., 2012), by affecting survival (Schaub, 1989; Maudlin et al., 1998; Anderson et al., 1999a; Joshi et al., 2002; Maciel-de-Freitas et al., 2011), reproductive performances (El Sawaf et al., 1994; Hogg and Hurd, 1995; Styer et al., 2007; Fellet et al., 2014; Vantaux et al., 2014), and sugar-seeking (Nyasembe et al., 2014), as well as probing and biting activities (Grimstad et al., 1980; Jenni et al., 1980; Wekesa et al., 1992; Platt et al., 1997; Anderson et al., 1999b; Koella et al., 2002; Botto-Mahan et al., 2006). For example, *Anopheles* mosquitoes infected with *Plasmodium* parasites, take larger blood meals (Koella and Packer, 1996; Anderson et al., 1999b), exhibit enhanced probing activities (Wekesa et al., 1992) and higher frequency of multiple blood feeding (Koella et al., 1998, 2002). Furthermore, *Plasmodium*-infected *Anopheles* females are more responsive to human skin odours, compared to their non-infected counterparts (Smallegange et al., 2013), and their likeliness to approach a host is dependent on the developmental stage of the parasite (Anderson et al., 1999b; Cator et al., 2013). In contrast, another study has shown that infected *An. coluzzii* display similar short- and long-range attraction to human odor, at both stages of infection, compared to non-

infected females (Vantaux et al., 2015). The minimum amount of blood ingested, required to induce host-seeking inhibition in *Ae. aegypti* infected with the avian malaria *P. gallinaceum*, was found to be higher during the sporozoite stage, when parasites become transmissible from the vector to humans (Koella et al., 2002). Thus, the infection by *Plasmodium* has been shown to impact host-seeking in malaria-infected mosquitoes, which consequently may have dire implications on the transmission of the disease to human hosts.

In comparison with malaria, little is known about the effects that NTD pathogens, particularly viruses, have upon host-seeking-related traits in the mosquito vectors. One of the first studies demonstrated that La Crosse virus-infected *Ae. triseriatus* females probed more frequently, while they engorged less than non-infected individuals, thereby increasing the risk of multiple feeding (Grimstad et al., 1980). Similarly, dengue-infected *Ae aegypti* females probe and feed longer, while being less frequently interrupted during blood feeding, suggesting a potential suppression of normal reflexes caused by the virus (Platt et al., 1997; Sylvestre et al., 2013). It should be noted that the behavioural modulation caused by the dengue virus, as reported in the literature, seems to be strongly dependent on the virus serotype. As an example, the capacity of locating a host and blood feeding, were found to be influenced by DENV-3 in *Ae. aegypti* (Platt et al., 1997), but not by DENV-2 (Putnam and Scott, 1995; Platt et al., 1997; Sylvestre et al., 2013). Locomotion, which is involved in a combination of behaviours, including host-seeking (Araripe Ordunha et al., 2018; Eilerts et al., 2018), has also been shown to be enhanced in dengue-infected *Ae. aegypti* during the two greatest peaks of the circadian activity (Lima-Camara, 2010). Similarly, a stage-specific modulation of host-seeking has been described in *Plasmodium*-infected mosquitoes (Anderson et al., 1999b; Koella et al., 2002; Cator et al., 2013). As such, understanding the processes underlying pathogen-induced alterations of behaviour and physiology in mosquitoes, is of great public health concern, and may contribute to the control of the transmission of vector-borne diseases.

## 2.4 Chemical ecology of host-seeking

Olfaction is the principal sense by which mosquitoes locate their host (Bowen, 1991). Host-seeking refers to the flight activation of a receptive mosquito and attraction towards its host in response to the emission of airbone airborne volatiles. While short-distance attraction is triggered by host heat and moisture, the long-distance attraction is influenced by visual and olfactory cues (Takken, 1991; Cardé, 2015). Although air-borne chemical cues, *i.e.* kairomones, have been shown to be involved in mosquito orientation and host location, much remains to be investigated regarding their identity. Mosquitoes are able to locate vertebrate hosts using volatile cues, such as carbon dioxide (henceforth abbreviated $CO_2$), lactic acid (LA) or 1-octen-3-ol, as well as detecting body heat and moisture (Kline et al., 1991; Takken and Knols, 1999; Zermoglio et al., 2017; Omondi et al., 2019). Besides $CO_2$, LA and 1-octen-3-ol, which are the most-studied mosquito attractants, additional host volatiles have been proven to be as just important in modulating host seeking (Cork and Park, 1996b; Bernier et al., 2000, 2002; Curran et al., 2005; Gallagher et al., 2008). Moreover, varation in the host odour profiles (Lacroix et al., 2005; Moraes et al., 2014; Kelly et al., 2015; Emami et al., 2017; De Moraes et al., 2018; Robinson et al., 2018), genotype (Verhulst et al., 2013), and blood type (Wood, 1974, 1976; Shirai et al., 2004; Anjomruz et al., 2014), strongly influence the degree of attractiveness of human beings to mosquitoes, and help mosquitoes to differentiate between individual human hosts (Lindsay et al., 1993; Knols et al., 1995; Bernier et al., 2002; Mukabana et al., 2002; Qiu et al., 2006a; Logan et al., 2008).

### 2.4.1 The role of semiochemicals in host-seeking

One of the main difficulties in identifying bioactive odorants regulating mosquito host-seeking is that odorant acts in a context-dependent manner, which is dependent on all of the other bioactive odorants present (Majeed et al., 2016, 2017; McBride, 2016; Omondi et al., 2019; Tallon et al., 2019). Several hundreds of VOCs have been detected in human emanations (Bernier et al., 2000; Curran et al., 2005; Penn et al., 2007; Gallagher et al., 2008), which have restricted the identification of bioactive odorants involved in the modulation of mosquito host-seeking (Smallegange et al., 2005; Qiu et al., 2011; Omondi et al., 2019)(Tallon et al. unpublished data). Thus, in order to

identify relevant semiochemicals involved in host-seeking, one must carefully consider several parameters, including: i. the nature and source of the compounds; ii. the emitted levels of compounds from the odour source; iii. the combination of different stimuli in time and space (Bowen, 1991).

Mosquitoes can react differentially according to the nature of the odorants encountered. For instance *An. gambiae* females are more sensitive to short chain carboxylic acids than 1-octen-3-ol and higher chain carboxylic acids (Meijerink and Van Loon, 1999). Non-host volatiles have been shown to repel host-seeking *An. arabiensis* (Jaleta et al., 2016), revealing that the source of emission is a key determinant of host-seeking. Aside from the presence/absence of certain compounds, the nature and position of the functional groups in an odorant molecule, *i.e.* different enantiomers may also modulate host-seeking. For instance, both *Ae. aegypti* and *Cx. quinquefasciatus* display a strong selectivity for the enantiomer (R)-(-)-1-octen-3-ol (Bohbot and Dickens, 2009; Grant and Dickens, 2011; Hill et al., 2015). The amount of volatiles emitted by a specific odour source are particularly important to know since different release rates of the same compounds can trigger distinct effects on mosquito behaviour (Carroll N. Smith, Nelson Smith, Gouck, Weidhaas, 1964; Gillies, 1980). Changes in the release rates of general host-related volatiles, within the naturally-encountered background of both host and non-host semiochemicals, *e.g.* 1-octen-3-ol and $CO_2$, can have dramatic effects in the evoked host-seeking behaviour (Majeed et al., 2016, 2017). Moreover, host-seeking is likely not only dependent on quantitative but also qualitative differences in mixtures of semiochemicals which enable odour-mediated behaviours in mosquitoes. It is generally assumed that host-seeking is elicited by complex compositions of multiple odorants rather than by a single compound, and that certain components act as activators and/or enhancers of responses to other cues (Qiu et al., 2011; Omondi et al., 2019). Therefore, both the chemical identity of relevant host volatiles, *i.e.* both the nature and the source of the compounds, as well as the amount and combinations of compounds emitted, are fundamental parameters involved in the modulation of host-seeking.

### 2.4.2 Host volatiles and attractants

Mosquitoes locate their hosts primarily through olfactory cues, including *e.g.*, $CO_2$, LA, ammonia, and carboxylic acids. Takken (1991) provided one of the first comprehensive lists of mammalian host attractants, including a complex set of odorants emitted from different body regions, and, where applicable, the names of the compounds eliciting behavioral responses in mosquitoes (Takken, 1991). Of these semiochemicals, $CO_2$ is the best known mosquito kairomone (Takken, 1991). Almost all haematophagous insects respond to this major component of the breath of vertebrate hosts (Gillies, 1980; Nicolas, 1989; Guerenstein and Hildebrand, 2008). As $CO_2$ is present in the exhaled breath of vertebrates, this compound reliably informs host-seeking mosquitoes of the presence of a potential host, and is required for the activation, orientation and alighting of mosquitoes to the detected hosts (Gillies, 1980; Healy and Copland, 1995; Dekker and Cardé, 2011). Carbon dioxide is considered as both a potent activator, as it elicits activation even at concentrations, close to ambient concentrations (Dekker et al., 2005), and strong attractant (Takken, 1991; Dekker et al., 2005), reflected by *e.g.* increased trap capture of many mosquito species in the field in the presence of CO2 (Kline et al., 1991). However, a minimum threshold of concentration of $CO_2$ at approximately 0.01% above ambient concentration, is required to activate *An. gambiae* (Healy and Copland, 1995), and *Ae. aegypti* (Eiras and Jepson, 1991). In *Ae. aegypti*, the exposure to $CO_2$ alone strongly activates mosquito flight, *i.e.* to increase the take-off rate and flight activity, and elicit probing behaviour (Eiras and Jepson, 1991). The nature of relevance of $CO_2$ during the process of host-seeking and host location varies however, from one species to another (Snow, 1970; Healy and Copland, 1995; Mboera et al., 1997; Dekker and Takken, 1998). For instance, the response to $CO_2$ differs between closely related anopheline species, in which zoophilic and opportunistic species are attracted to higher amounts of $CO_2$, compared to the more anthropophilic species, which coincides with the release rates of $CO_2$ from their respective hosts (Dekker and Takken, 1998). The importance of this kairomone was shown to be age-dependent, corresponding to the development of female host-seeking (Grant and O'Connell, 2007; Omondi et al., 2015a).

Carbon dioxide act as a synergist (Eiras and Jepson, 1991; Kline et al., 1991; Becker et al., 1995; Takken et al., 1997; Geier and Boeckh, 1999; Dekker et al., 2005). For instance, LA and human sweat samples only

elicit take-off and probing behaviour in *Ae. aegypti*, when they are supplemented with $CO_2$ (Eiras and Jepson, 1991). Interestingly, the action of $CO_2$ was shown to be species-specific when tested in the field, where *e.g.*, the effect of $CO_2$ combined with 1-octen-3-ol was additive in *Culex* spp. and *Diachlorus ferrugatus*, while the effect was synergist in *Ae. tarniorhynchus* (Kline et al., 1991). In *Ae. aegypti*, $CO_2$ has also been shown to be required for the mediation of the response to heat and LA (McMeniman et al., 2014), and increases the sensitivity and responsiveness to skin odours, suggesting an instantaneous behavioural sensitization of the olfactory circuitry by $CO_2$ (Dekker et al., 2005). This behavioural synergism is suggested to occur centrally, and not at the receptor level (Davis and Sokolove, 1976).

Since $CO_2$ on its own, accounts for a minor part of the overall long-range attractiveness, particularly in anthropophilic species, this suggests the involvement of additional semiochemicals in mosquito host-seeking (Mboera et al., 1997). Moreover, while zoophilic mosquitoes, tend to land and bite more on the face, more specialist feeder mosquitoes, such as *A. gambiae,* preferentially bite on different body parts depending on the position of the hosts (Dekker et al., 1998) This study suggests a lesser involvement of $CO_2$ in host-seeking behaviour. while convection currents, together with the emission of host cues, can guide mosquito biting site selection (Dekker et al., 1998). Various studies investigating the production of host-emitted odorants functioning as kairomones for mosquitoes, have revealed that extracts from human emanations, contained both LA and ammonia (Geier et al., 1999), as well as hundreds of compounds including carboxylic acids, alcohols, esters, aldehydes, aliphatics, aromatics and ketones (Cork and Park, 1996b; Bernier et al., 2000, 2002; Curran et al., 2005; Gallagher et al., 2008). The first group of compounds demonstrated to be attractive for *An. gambiae* consisted of a synthetic mixture of aliphatic carboxylic acids (Mboera et al., 1997). Later, 1-octen-3-ol was identified as a strong olfactory attractant for tsetse flies when emitted from cattle (Hall et al., 1984). Subsequently mosquitoes have been shown to be responsive to (*R*)-1-octen-3-ol (Cork and Park, 1996b; Qiu et al., 2006b; Lu et al., 2007). While the perception of 1-octen-3-ol within a blend likely plays a vital role in host selection and discrimination in a range of mosquito species (Majeed et al., 2016), this compound does not appear to be the main host finding cue, since this compound alone does not elicit any effect in wind tunnel experiments (Takken et al., 1997), nor does it increase trap catches in the

field (Njiru et al., 2006). The collection and identification of host emanations and their incorporation into synthetic odour blends allow to deliver odorants in natural emission ratios, which have been shown to trigger host-seeking in mosquitoes (Mathew et al., 2013; Busula et al., 2015; Omondi et al., 2019).

Individual components of a host blend are often not recognized as host cues when presented outside the context of the mixture (Geier et al., 1999; Braks et al., 2001; Qiu et al., 2011; Majeed et al., 2016). The development of odour-based lures has led to successful trapping strategies in the field (Smallegange et al., 2009; van Loon et al., 2015; Homan et al., 2016), however, it has been emphasised that the use of these odour blends in their natural release rates and proportions may further enhance their trapping efficiency (Majeed et al., 2016; Ghaninia et al., 2019). For instance, mosquitoes are capable of detecting variations in the emission of host-related odorants at the release rates of nanogram to subnanogram per minute (Majeed et al., 2016; Ghaninia et al., 2019). The combination of presence/absence of compounds, and their association in odour blends is therefore an essential element of mosquito host-seeking. Certainly, future investigations, *e.g.* odour collection, volatile identification, behavioural and physiological assay, are continuously required to better understand the role of host volatiles in the modulation of host-seeking behaviour in disease vectors.

### 2.4.3 Inter-individual variation in human attractiveness

Mosquitoes are effective at differentiating between individual human beings, who can be ranked for attractiveness to mosquitoes based on their odour profiles (Lindsay et al., 1993; Knols et al., 1995; Bernier et al., 2002; Mukabana et al., 2002; Qiu et al., 2006a; Logan et al., 2008). The presence of individual and gender fingerprints in human odour profiles is widely accepted and investigated (Curran et al., 2005; Zhang et al., 2005; Penn et al., 2007). Moreover, certain VOCs have been proposed to be chemical signature markers of the age of individuals as well as of specific body parts (Gallagher et al., 2008). Injured, diseased or malnourished hosts may also emit different VOCs, as mosquitoes have been speculated to be able to modify their host-seeking behaviour on these hosts, which provide a poorer quality blood meal than healthy individuals (Kelly, 2001), which may affect

the mosquito fitness (Logan, 2008). Some reports also say that mosquitoes can respond differentially depending if their host is pregnant (Ansell et al., 2002; Himeidan et al., 2004), or diseased as these physiological conditions are associated with altered odor emissions (Lacroix et al., 2005; Moraes et al., 2014; Kelly et al., 2015; Emami et al., 2017; De Moraes et al., 2018; Robinson et al., 2018). In these particular cases, investigating the causes underlying changes in VOCs emitted by the hosts, may contribute to a better knowledge of the alteration of mosquito host-seeking behaviour.

The analysis of odour profiles between individuals differing in their attraction of mosquitoes, have permitted the identification of compounds from a broad range of chemical classes likely influencing mosquito host discrimination (Bernier et al., 2002; Verhulst et al., 2013; Robinson et al., 2018; Wooding et al., 2020). For instance, the production of free fatty acids, which are among the most abundant human skin surface lipids (Nicolaides, 1974), has been demonstrated to be linked to the metabolic activity of skin microorganisms and differential attractiveness levels of humans to mosquitoes (Knols et al., 1995). Several factors may explain the differences observed between individual odour profiles, *e.g.* the diversity and abundance of skin bacteria (Braks et al., 2001) commonly associated with body odour (MorettiI, 1960; Grice et al., 2008), and which has been shown to influence host-seeking (De Jong and Knols, 1995), as well as the variation in volatiles emitted by different communities of human skin microorganisms (Ara et al., 2006; Xu et al., 2007; Verhulst et al., 2009, 2010). For instance, human individuals ranked as highly attractive to *An. gambiae* females have been shown to present higher abundance of skin bacteria, while lower diversity, than individuals considered poorly attractive (Verhulst et al., 2011). Furthermore, specific compounds were shown to be involved in inter-human individual variation in attractiveness, in which *e.g.*, aldehydes were demonstrated to be released in relatively greater amounts from the skin of *Plasmodium*-infected hosts, who were significantly more attractive to mosquitoes (Robinson et al., 2018). These aforementioned compounds may serve as biomarkers of malaria or be used to enhance the efficacy of chemical lures used to trap mosquitoes (Verhulst et al., 2011). Further investigation is needed regarding the implications of such volatiles in the mediation of host-seeking, which would contribute in the development of novel odour-baited trapping systems based on such host semiochemicals (Kline, 2007).

Body odours carry information regarding the genetic identity and similarities between individuals (Roberts et al., 2005). The genetic background of human body odour profiles is partly determined by the major histocompatibility complex genes implicated in the human immune response, including the human leukocyte antigen (HLA) alleles that are involved in regulating body odour and mating preferences in humans (Penn and Potts, 1999; Jacob et al., 2002). Interestingly, a correlation has been described between individuals carrying HLA-Cw alleles and a high level of attraction to malaria mosquitoes (Verhulst et al., 2013). However, little information is available regarding the association between inter-individual variation in human odour profiles and their HLA profile, and how this relates to their level of attractiveness of mosquitoes. Besides the implication of the major histocompatibility complex genes in the inter-individual variation in human attraction to mosquitoes, blood type has also been shown to strongly influence the degree of attractiveness of human beings (Wood, 1974, 1976; Shirai et al., 2004; Anjomruz et al., 2014). Of note, early experiments showed that the ABO status of the blood meal taken by mosquitoes is a reliable marker to determine the blood group of the bitten individual (Wood, 1976; Bryan and Smalley, 1978). From these observations, the blood type of the human host was suggested to contribute to the inter-individual variation in attractiveness to mosquitoes, in which both *An. gambiae* and *Ae. aegypti* demonstrated a strong preference for humans with blood group O (Wood, 1974, 1976). Similarly, *Ae. albopictus* females tend to be more attracted towards this same blood type, when compared to the others (Shirai et al., 2004). However, one should keep in mind that these observations are correlative and may very likely be context-dependent. However, the preference of mosquitoes for a certain blood type appears to be dependent on the geographical region, and researchers have speculated that this acts as a natural selection pressure to maintain the ABO blood factor polymorphisms among human populations, particularly where the risk of vector-borne diseases is considerable (Wood, 1974, 1976; Anjomruz et al., 2014). For instance, *An. stephensi* exhibit higher preferences towards blood type AB in the malaria-endemic regions of south of Iran and India, where the prevalence of this blood group is low (Anjomruz et al., 2014). Understanding the relationship between inter-human individual variation in attractiveness to mosquitoes and differences between specific body odour profiles, genotypes, and/or blood type is necessary for further identification of compounds which

may offer potential for the development of improved intervention strategies against disease vectors.

**BOX 2. Dengue, currently the most rapidly spreading vector-borne disease in the world**

Information of about the global distribution and burden of NTDs, including dengue, is generally scarce and not well-documented. The incidence of dengue, estimated to 100-400 million infection in hundreds of tropical and subtropical regions of the world each year, had expanded exponentially around the world in the past two decades (Brady et al., 2012; WHO, 2012; Bhatt et al., 2013; World Health Organization, 2017b), and the total epidemiological and global economic burden of the disease is estimated at US$ 8.9 billion annually (Shepard et al., 2011, 2014, 2016). Dengue is massively under-reported (Shepard et al., 2016; World Health Organization, 2017b), with recent reports suggesting a three-times higher infection rate than previously predicted (World Health Organization, 2009; Bhatt et al., 2013; WHO, 2015). Even though, most of the infection burden is in Southern East Asia, Latin America, and Western Pacific regions (Bhatt et al., 2013; World Health Organization, 2017b), several African countries are found with endemic dengue, or with high probability for the virus to occur (Jaenisch et al., 2014; Agha et al., 2017; Lim et al., 2018). However, dengue is still fairly undocumented in the African continent. Following local variations of rainfall, temperature, and uncontrolled urbanization (Ebi and Nealon, 2016; World Health Organization, 2017b), the distribution of dengue throughout the tropics coincides with the occurrence of its main vectors, *Ae. aegypti* female mosquitoes, and to a lesser extent, *Ae. albopictus* (Bhatt et al., 2013; Agha et al., 2017). The rapid and ongoing geographic expansion of these two vectors, which are exceptionally adaptable and anthropophilic, strongly influences dengue infection burden in these regions (Black IV et al., 2002; Salazar et al., 2007; Takken and Knols, 2007; Kyle and Harris, 2008; Scott and Takken, 2012; Fischer et al., 2014; Kraemer et al., 2015b; Akiner et al., 2016). Interestingly, natural cases of transovarial (vertical) transmission of dengue, from infected females to their offspring, have

been reported, which was speculated to be a possible novel strategy of coping with challenging environmental conditions, *i.e.* high temperatures and low human densities (Günther et al., 2007; Arunachalam et al., 2008; Le Goff et al., 2011; Gutiérrez-Bugallo et al., 2017). Furthermore, venereal (horizontal) transmission, between infected mosquitoes of both sexes, was shown to be possible under laboratory conditions (Putri et al., 2018), highlighting the potential role of mosquitoes in maintaining dengue in local populations and revealing that their efficiency in transmitting the virus is likely largely underestimated.

Climatic changes, associated with globalisation, increase the burden of dengue in endemic areas (Martens et al., 1997; Epstein, 1999), as well as the risk of outbreaks in the Northern hemisphere (Hofhuis et al., 2009; Kolivras, 2010; Ebi and Nealon, 2016; Butterworth et al., 2017). Since the first notice of local cases of dengue in southern Europe in 2010, autochthonous infections have been sporadically observed every year (Jelinek, 2008; Rezza, 2012), and local transmissions are likely to become evident and frequent in certain regions of Europe, following the return of infected travellers from dengue endemic regions (Allwinn et al., 2008; Massad et al., 2018). In 2019, which was the year of the worst global outbreak ever reported, Brazil on its own registered an increase of cases by 488 % (World Health Organization, 2019; Papaleo, 2020), WHO had included dengue in its list of the world's top 10 public health threats. The growing problem of dengue outbreaks is particularly noticeable this year, when in the shadow of coronavirus, the alarming increase in infections continues to take shape (Papaleo, 2020; World Health Organization, 2020).

Dengue fever is characterized by a wide spectrum of symptoms, from subclinical disease (asymptomatic patients) to severe flu-like illness and intense joint pain, and in some cases, severe complications leading to serious bleeding, organ impairment and/or plasma leakage. The risk of dengue haemorrhagic fever (DHF) represents a major global public health problem, especially when not managed appropriately in the case of resource-poor regions, generally lacking relevant diagnostic and surveillance tools, healthcare settings and adapted policies (Bhutta et al., 2014). Until now, four distinct, although closely related, dengue (DENV) serotypes (DENV-1, DENV-2, DENV-3 and DENV-4), belonging to the Flaviviridae family, have been identified to be responsible for dengue

(World Health Organization, 2017b). While several countries are hyper-endemic for all four DENV serotypes (9; https://nextstrain.org/dengue/denv1?c=region for a real-time tracking of dengue serotype evolution), temporal variation in prevalence of the different serotypes coincides with the epidemic emergence of dengue in certain regions of the world (Ocazionez et al., 2006; Sasmono et al., 2018). While the recovery from infection by one particular serotype provides protection against a subsequent infection with the homologous serotype, consecutive infections by heterotypic serotypes likely increase the probability to develop severe DHF, as cross-immunity to the other serotypes after recovery is only partial and temporary (World Health Organization, 2017b). Despite several attempts to design tools *e.g.* plant-synthesized silver nanoparticles (Murugan et al., 2016), and the development of a controversial vaccine (Dengvaxia®) in 2015, targeting people of a certain age range and living in endemic regions (World Health Organization, 2017a), there is currently no specific treatment for the disease.

Following the uptake of blood from a viremic person, a mosquito becomes infected when the virus enters the midgut and makes contact with the cells of its epithelium. As the virus replicates within these cells, and reaches the hemocoel, secondary tissues, including the salivary glands, become subsequently infected, and the mosquito becomes infectious, *i.e.* capable of transmitting the virus via its saliva to a new human host during the next blood meal (Carrington and Simmons, 2014). The viral titres circulating in the blood of an infected person required for the vector to become infected, varies across the serotypes and depends on the type of infection, in which the infectious dose necessary to infect 50% of a population of mosquitoes is ca. 10-fold lower with DENV-1 and DENV-2 than with the two other serotypes (Nguyen et al., 2013), and ca. 100-fold lower in patients with asymptomatic and presymptomatic infections (Duong et al., 2015). In humans, the intrinsic incubation period, *i.e.* the time between a human is being bitten and the onset of symptoms due to the infection, varies between 1 and 8 days and a person can be viremic from up to 2 days prior the development of symptoms, for 1 to 12 days after the onset of illness, and until up to 2 days after the fever has resolved (Chan and Johansson, 2012; Carrington and Simmons, 2014). Notably, variations in the extrinsic incubation period (EIP), *i.e.* the viral

incubation period from ingesting the virus to when the mosquito becomes infectious (Kyle and Harris, 2008; Chan and Johansson, 2012), were found between infected tissue (Linthicum et al., 1996; Platt et al., 1997; Salazar et al., 2007; Raquin and Lambrechts, 2017), and shown to be dependent on ambient temperatures and viral concentrations (McLean et al., 1975; Watts et al., 1987; Carrington et al., 2013). In mosquitoes, vector competence was also shown to be strongly dependent on the interaction between the virus and vector genotypes (Vazeille et al., 2003; Lambrechts et al., 2009).

**BOX 3. Other neglected tropical diseases transmitted by *Ae. aegypti***

In addition to dengue fever, *Ae. aegypti* transmit other major arboviral diseases including yellow fever (YF), chikungunya virus (CHIKV), and Zika (ZIKV) (Leta et al., 2018). These NTDs represent a global public health threat of varying magnitude, as 123 countries/territories have reported more than one of the aforementioned diseases (Leta et al., 2018). On average, autochthonous cases of Zika, chikungunya, and YF have been disclosed in 85, 106, and 43 countries, respectively (Leta et al., 2018). YF is mainly distributed across tropical regions of South America and sub-Saharan Africa, and despite the availability of an effective vaccine since 1938, this flavivirus remains a continuing threat to travellers and residents of endemic areas (Monath, 2005; Monath and Vasconcelos, 2015) There is ca. 200 000 cases and 30 000 deaths from YF reported worldwide per year, 90 % of them in Africa (Monath and Vasconcelos, 2015). While humans (secondary hosts) generally become infected after being bitten by an infected female mosquito that has acquired the infection from monkeys (primary hosts), through the sylvatic or intermediate transmission cycle, inter-human transmission may also occur, through the urban transmission cycle when infected people introduce the virus into regions with high density of mosquitoes and humans (Monath and Vasconcelos, 2015). Once transmitted, the general incubation time of YF in the body of the host is 3-6 days, and the most common symptoms are fever, muscle pain particularly in the back, headache, loss of appetite, and nausea or vomiting, which can deteriorate into a severe fever and liver and kidney failure (Porudominsky and Gotuzzo, 2018).

Both CHIKV and ZIKV are among the most recent mosquito-borne viruses introduced into the Americas (Fauci and Morens, 2016b; Shragai et al., 2017). Similar to YF and dengue, ZIKV belongs to

the Flaviviridae family (Shragai et al., 2017). Compared to YF, which is described as the first recognized viral cause of the deadly epidemics associated with hemorrhagic fever (Fauci and Morens, 2016a), ZIKV has been isolated in a sentinel Rhesus monkey during a surveillance program of YF in the Zika forest of Uganda in 1947 (Dick et al., 1952). ZIKV only became considered a major public health concern due to the recent pandemic of ZIKV infection in Latin America in 2016, in which at least 360 000 cases were suspected. This outbreak highlighted the potential threat by this arbovirus in the northern hemisphere (Fauci and Morens, 2016a; Shragai et al., 2017). CHIKV is another example of an arbovirus that has significantly expanded its geographic range worldwide over the last decades (Mayer et al., 2017). CHIKV, likely originate from sub-Saharan Africa, where it may have been initially maintained in the region through enzootic transmission cycle between non-human primates and arboreal *Aedes* mosquitoes, and then introduced into Asia (Volk et al., 2010). Unlike the other aforementioned arboviruses, CHIKV is an alphavirus of the Togaviridae family. While mortality is relatively low, the morbidity is high especially in particular population groups, *e.g*. in babies, elderly and immunocompromised patients, in which CHIKV infection is characterised by a sudden onset of high fever, headache, skin rash, as well as joint and muscle pain which may last from several weeks to several years (Borgherini et al., 2008). Similarly to ZIKV, CHIKV was first identified as an endemic disease in East Africa in 1952 (Schwartz and Albert, 2010), while nowadays CHIKV is present in more than 45 countries, with over 1.7 million suspected cases annually (https://www.who.int/news-room/fact-sheets/detail/chikungunya). The incidence and prevalence of both CHIKV and ZIKV are difficult to estimate among human populations since, i. a high proportion of infected persons are asymptomatic, and ii. reliable techniques to diagnose these diseases are limited or expensive in most endemic areas (Shragai et al., 2017). Along with the bite from an infectious female mosquito, these arboviruses can be transmitted maternally, through blood transfusion, and, in the case of Zika, through sexual contact (Musso et al., 2014; D'Ortenzio et al., 2016). Worryingly, *Ae. aegypti* has been shown since long to be able to transmit YF vertically (Diallo et al., 2000), implying higher transmission risks and outbreaks. Similarly, transovarial transmission of Zika has been shown to be possible in mosquitoes (Li et al., 2017). Unlike YF, no vaccine is available for these NTDs.

# 3. Mosquito peripheral olfactory system

## 3.1 Peripheral olfactory organs

The head of most adult dipterans, including mosquitoes, is equipped with three types of olfactory organs, including the antennae, and the mouthparts, the maxillary palps and proboscis, which act as secondary olfactory organs (Hildebrand and Shepherd, 1997; Touhara and Vosshall, 2009; Hopf et al., 2015). The tremendous task of locating and detecting biologically-relevant volatiles is mediated by olfactory receptor neurons (ORNs), which are located in porous sensory hairs, *i.e.* sensilla, covering the surface of these olfactory organs (McIver, 1982; Hansson, 2002; Bruce et al., 2005; Su et al., 2012). Although sensilla are the organs where chemical, but also other sensory stimuli, including *e.g.*, mechanical and thermal stimuli are detected (Stocker and Gendre, 1988; Clyne et al., 1997; Yao et al., 2005; Su et al., 2012), this thesis emphasises on olfaction.

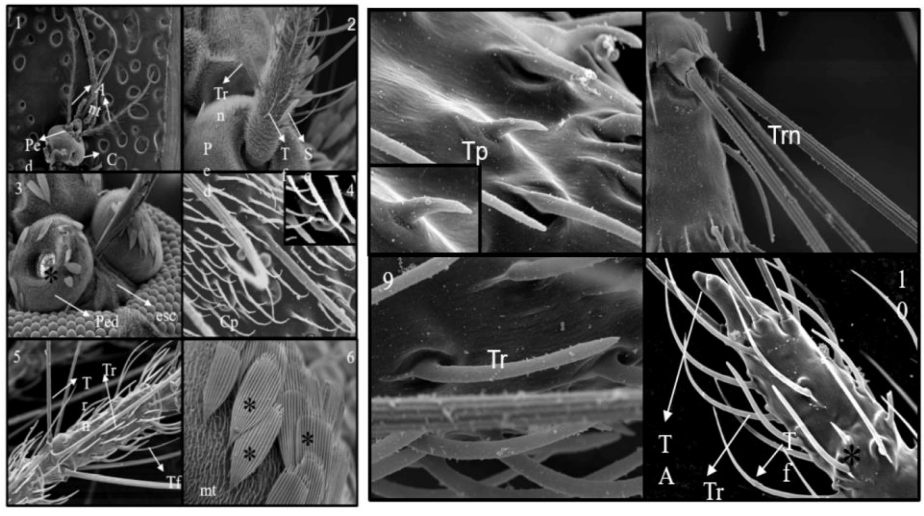


Figure 1. Peripheral olfactory organs and chemosensory sensilla of female *Ae. aegypti*. Courtesy of Paulo F. P. Pimenta (Fiocruz, Manaus, Brazil). General view of the head (C) showing the pedicel (Pe) located at the base of the antenna (Ant) (1-3). The antennae are covered by the non-olfactory sensilla, sensilla ampullacea (sq) (2,6), and the olfactory sensilla, sensilla coecolonica (Cp) (4) and sensilla trichoid (Tm, Tf) (2,5,7-9).

### 3.1.1 Main olfactory organ – the antenna

In mosquitoes, both adult females and males possess antennae constituted of 15 segments, including a ring-shaped scape, a globular pedicel, and 13 flagellar segments (Figure 1) (Steward and Atwood, 1963; McIver, 1978, 1982). However, an important sexual dimorphism exists regarding the morphology of this organ, in that olfactory sensilla are on average twice as numerous in females as in males, and are generally restricted to the distal segments in male antennae (Roth and Willis, 1952; McIver, 1982). The sex-related discrepancy in the number of chemosensilla present in the antennae might be linked to sex-specific behaviours. In addition, most male mosquitoes possess a larger number of long and shaggy sensilla, called fibrillae, present on each flagellar segments, which gradually get erected around the first 48 h post emergence, as mosquitoes become sexually mature (Gibson et al., 2010). Noteworthy, a hearing organ, *i.e.* Johnston's organ, located at the base of each antenna of both sexes, is likely involved in antennal responsiveness to specific frequency of flight sounds, and was

suggested to play an essential role in mate location and species recognition (Göpfert and Robert, 2000; Cator et al., 2009; Gibson et al., 2010). In *Ae. aegypti*, this organ was also described to participate in in-flight manoeuvres towards host odours, acting as a flight stabilizing sensor following an encounter with $CO_2$ (Dekker and Cardé, 2011). Later, the antennae were confirmed to be the main olfactory organs in mosquitoes, involved in host detection (Roth, 1951; Steward and Atwood, 1963; Maekawa et al., 2011), as well as in moisture and warmth sensing (Roth and Willis, 1952; Kellogg, 1970). In addition, host cues were found to be perceived by sensory cells located on the antennae (Takken, 1991; Meijerink and Van Loon, 1999).

### 3.1.2 Other olfactory appendages

The maxillary palp is mainly involved in olfaction and mechanoreception, and has a simpler structure than the antenna, containing less sensilla (Vosshall and Stocker, 2007). In mosquitoes, the maxillary palps, are constituted of five segments, of which the most distal differs in shape between sexes and across species (McIver, 1982). The maxillary palps are involved in the detection of a wide range of volatiles, including *e.g.*, host cues and plant-derived compounds, suggesting a broad spectrum of possible functions for this organ (Kellogg, 1970; Syed and Leal, 2007). The maxillary palps contain specialized $CO_2$-ORNs (Kellogg, 1970; Grant et al., 1995; McMeniman et al., 2014; Omondi et al., 2015a) capable of sensing rapid changes and low $CO_2$ concentrations, independent of the background level present in the environment (Kellogg, 1970; Grant et al., 1995). The removal of the maxillary palps was shown to lead to a drastic reduction in the ability to locate a host in *An. stephensi* (Maekawa et al., 2011). Interestingly, the sensitivity of $CO_2$-receptors expressed in the maxillary palps was shown to coincide with the onset of host-seeking in *An. gambiae* (Omondi et al., 2015a), and host preferences among mosquito species (Majeed et al., 2017). Furthermore, other host-related compounds, such as 1-octen-3-ol, can be detected by ORNs housed in sensilla located on the maxillary palps, therefore emphasising the potential implication of this organ in mosquito host-seeking (Grant and Dickens, 2011).

The proboscis, initially thought to only detect non-olfactory cues *i.e.* taste and mechanical stimuli, has also been demonstrated to have an olfactory function in mosquitoes (Kwon et al., 2006; Maekawa et al., 2011; Won Jung

et al., 2015). In haematophagous insects, proboscides are essential mouthpart appendages consisting of long and thin stylet-shaped structures that have evolved to puncture the skin of their hosts (Krenn and Aspöck, 2012). In mosquitoes, the piercing structures (labrum, mandibles, laciniae and hypopharynx) lie within the labium which bends backward outside the skin of the host when the insect is probing (Krenn and Aspöck, 2012). This piercing-sucking organ possesses sensory hairs housing ORNs that express a subset of olfactory receptors (Kwon et al., 2006; Maekawa et al., 2011; Sparks et al., 2014; Won Jung et al., 2015). Unlike the ORNs located in the maxillary palps which are likely involved in the long-range orientation towards hosts, the ORNs present in the proboscis were speculated to play a key role in the final stage of host-seeking, *e.g*. skin penetration and blood vessel targeting (Won Jung et al., 2015; Riabinina et al., 2016). Moreover, proboscides are likely not involved in the detection of $CO_2$ (Maekawa et al., 2011). Furthermore, both the labellum and proboscis do likely not only function as a tool for food intake but also play a fundamental role in host-seeking and heat recognition in mosquitoes (Kwon et al., 2006; Maekawa et al., 2011; Won Jung et al., 2015), *e.g.* ORNs identified in the labellum and the proboscis of both *An. gambiae* and *Ae. aegypti* were suggested to be involved in the integration of olfactory signals (Won Jung et al., 2015; Riabinina et al., 2016) Moreover, chemosensory-related receptors have been found to be expressed in the proboscis (Bohbot et al., 2007b; Maekawa et al., 2011). In mosquitoes, the two labellar lobes located at the tip of the labium are required for feeding behaviour (Clements, 1999), and possess a subset of ORNs that has been showed to display robust olfactory responses (Kwon et al., 2006), and gustatory receptor neurons (GRNs) (Kwon et al., 2006; Sparks et al., 2013a, 2014), as well as chemosensory receptors involved in the detection of repellent chemicals, such as DEET (DeGennaro et al., 2013).

### 3.1.3 Chemosensory sensilla

Chemosensory sensilla are sensing organs consisting of outer sheath cells, bipolar ORNs, and cuticular supporting cells (McIver, 1982). When odorant signals reach the antennae of an insect, the odorants cross the porous cuticular walls of the sensilla, and are transported through the sensillar lymph surrounding the ORNs (Hildebrand and Shepherd, 1997; Ignell et al., 2010). The recognition of the external stimuli is made through these ORNs.

Mosquitoes have developed very sophisticated antennae structures housing a considerable amount of sensilla, *i.e.* ca. 90 %, involved in olfaction, taste, mechano-, hygro-, and thermoreception, which vary in size and abundance between sexes and among species (McIver, 1971, 1982; Keil, 1999; Gibson et al., 2010; Hill et al., 2010). In mosquitoes, there are two major morphological types of sensilla, single- and double-walled, and five different classes of sensilla, including the olfactory sensilla: the thin and double-walled coeloconica, basiconica (pegs) and trichoidea (hairs) sensilla, as well as the non-olfactory sensilla: the double-walled chaetica (bristles) and the elliptical-shaped ampullacea sensilla (Figure 1) (McIver, 1982; Pitts and Zwiebel, 2006; Hill et al., 2009). Distributed evenly across the segments 2–13 of the antenna, sensilla chaetica are sharp-pointed tipped, articulated bristles arising from sturdy sockets, and the largest sensilla described in mosquitoes (McIver, 1972; Seenivasagan et al., 2009). These sensilla function as mechanoreceptors and are proposed to contribute to the detection of air currents and orientation towards odour sources, with one particular subtype, *i.e.* antennal fibrillae, suggested to have a role in mate location and species recognition (McIver, 1972; Charlwood and Jones, 1980; Seenivasagan et al., 2009; Gibson et al., 2010). The sensilla coeloconica are innervated by three ORNs, and appear as tubular structures located on the distal tip of the antennae (McIver, 1973; Seenivasagan et al., 2009). This type of sensilla was shown to possess receptors sensitive to temperature changes (McIver, 1973; Davis and Sokolove, 1975), *e.g.* receptors expressed in the small sensilla coeloconica of *An. gambiae* were shown to be excited by the increase in air temperature (Wang et al., 2009), suggesting that these sensilla could play a role in the perception of heat emitted from hosts. The sensilla basiconica, and sensilla trichoidea are innervated by 3-5 and 1-2 ORNs, respectively, while the sensilla trichoidea are the most abundant class of sensilla, widely distributed along all the segments of mosquito antennae (Steward and Atwood, 1963; McIver, 1974, 1978, 1982; Hill et al., 2009). Both of these classes of sensilla can be separated under distinct morphological and functional subtypes (Bowen, 1995; Ghaninia et al., 2007, 2008; Hill et al., 2009; Seenivasagan et al., 2009). In general fewer sensilla of both classes were found in males than females, and *Culex* mosquitoes has been reported to consistently possess more sensilla basiconica than *Aedes* and *Anopheles* (McIver, 1971, 1982). Although sensilla basiconica and sensilla trichoidea have been reported as potential hygroreceptors (Kellogg,

1970; McIver, 1978), they mainly function as olfactory chemosensilla (Steward and Atwood, 1963; McIver, 1978), which house ORNs sensitive to various host-associated cues (Davis and Sokolove, 1976; Davis, 1977, 1984; Hildebrand et al., 1996; Meijerink and Van Loon, 1999; Ghaninia et al., 2007, 2008; Hill et al., 2009; Syed and Leal, 2009), including *e.g.*, CO2 (Lu et al., 2007), 1-octen-3-ol (Hill et al., 2015), as well as carboxylic acids (Hill et al., 2009) and chemicals associated with plant-related (Davis, 1977, 1988; Ghaninia et al., 2008; Hill et al., 2009) and oviposition site-related cues (Davis, 1977, 1988; Ghaninia et al., 2007; Hill et al., 2009; Syed and Leal, 2009). In mosquitoes, while short sensilla basiconica house ORNs sensitive to LA, long sensilla basiconica have been found to lack these LA-excited cells (Bowen, 1995). Similarly, in *Ae. aegypti*, the short sensilla trichoid respond to the extract of the plant species *Nepeta faassenii*, known as a mosquito repellent (Amer and Mehlhorn, 2006), whereas none of the main components of this plant extract elicited responses in the long sharp sensilla trichoid (Ghaninia et al., 2008). Interestingly, the differences in length and structure of sensilla observed between Culicinae species, were suggested to be linked to distinct mechanisms employed in the perception of chemicals between these species (Seenivasagan et al., 2009).

Over time, multiple studies have helped gain insight regarding the chemosensory organization and function of the peripheral olfactory organs which have an essential role in enabling specific vector-borne disease behaviours including host-seeking, blood feeding, and oviposition. Further functional characterization of olfactory sensilla remains necessary for a better understanding of how mosquitoes detect and exploit sensory cues implicated in enhanced disease transmission.

## 3.2 Chemosensory binding proteins

The foundation of the sophisticated olfactory system in insects relies on the ability of the peripheral olfactory system to detect considerable amounts of odorant molecules, by binding, transporting transducing and rapidly degrading the signals once they have conveyed information. In this pathway, there are several types of chemosensory proteins, each of which is involved in specific tasks. The binding and transport of odorants requires the involvement of an array of chemosensory proteins including odorant binding proteins (OBPs) (Wogulis et al., 2006; Leal et al., 2008), and chemosensory

proteins (CSPs) (Lartigue et al., 2002; Pelosi et al., 2006). It is proposed that all these proteins greatly contribute to the sensitivity of the olfactory system in insects. However, despite the efficient identification of candidate odorant molecules, and the emergence of techniques to express genes in heterologous systems, the specificity of recognition and transport by chemosensory binding proteins in the sensillar lymph remains poorly understood.

### 3.2.1 OBPs

Once the odorants have crossed the cuticle through the sensillar pores and reached the aqueous sensillum lymph, they are recognized and transported to the dendritic surface of the ORN by OBPs (Sánchez-Gracia et al., 2009). Odorant binding proteins are essential in making the liaison between the external environment and the chemoreceptors (Hekmat-Scafe et al., 2002; Rinker et al., 2013b; Matthews et al., 2016a; Tallon et al., 2019). Insect OBPs are small globular water-soluble proteins (ca. 135–220 residues in length) that bind, solubilise and transport hydrophobic odorant molecules to odorant receptors (ORs), and possibly other olfactory receptors expressed on the surface of the ORNs (Pelosi and Maida, 1995; Pelosi et al., 2006; Sánchez-Gracia et al., 2009). These proteins are secreted in large amounts by support cells that surround the ORNs (Hansson and Stensmyr, 2011), and are found in high concentrations in the sensillum lymph in both olfactory and non-olfactory tissues (Hekmat-Scafe et al., 2002; Biessmann et al., 2005; Li et al., 2005, 2008; Chintapalli et al., 2007; Sengul and Tu, 2008; Matthews et al., 2016b) Here, OBPs play important roles in the perireceptor events which precede the initiation of second messenger signalling cascade (Pelosi, 1996; Kaissling, 2009). Of note, OBPs are the chemosensory-related gene family detected with the highest transcript abundance in mosquito antennae (Pitts et al., 2011; Matthews et al., 2016b; Tallon et al., 2019), alike in other insects (Shiao et al., 2013; Latorre-Estivalis et al., 2017), suggesting an important role in the modulation of odour-mediated behaviours.

Although insect OBPs share similar functions to the OBPs described in vertebrates, these proteins bear no sequence homology to each other, and insect OBP sequences are generally much more highly conserved (Pelosi and Maida, 1995; Pelosi, 1996). Odorant binding proteins can be identified through their typical and well-conserved hydrophobic cavity covered by a six cysteine motif connected through disulfide bridges (Pelosi

and Maida, 1995). This class of proteins was first discovered in Lepidoptera in 1981 (Vogt and Riddiford, 1981). Since then, families of OBPs, with a highly varying number ranging from one to several hundreds have been described across insect species, although the number of these proteins and their function as OBPs in binding odorants in insects remains only partly understood (Hekmat-Scafe et al., 2002; Leal, 2004). The total number of OBPs identified in the mosquitoes *An. gambiae*, *Ae. aegypti* and *C. quinquefasciatus* is 69, 111, and 109, respectively (Manoharan et al., 2013). Within the same mosquito species, three subfamilies of OBPs can be discriminated according to their amino acid sequences, and the variation in tissue-specific expression; the Classic OBPs, the PlusC OBPs, and the Two-domains known as duplex OBPs and the atypical OBPs, whose structure, i.e. two-domain OBPs, differs from the conserved six cysteine motif (Manoharan et al., 2013). Comparative modelling of the structure of OBPs has helped gain insights into the contribution of these proteins in the mosquito olfactory system.

The compact structure of OBPs, *i.e.* α-helical domains interlocked by disulfide bridges, forms a conical-shaped hydrophobic cavity for odorant-binding (Zhou, 2010). In Lepidopterans, OBPs have been demonstrated to undergo a pH-dependent conformational change that determines ligand binding and release (Lautenschlager et al., 2005; Leal et al., 2005b, 2005a). In mosquitoes, *e.g.* AaegOBP1 of *Ae. aegypti* (Leite et al., 2009), AgamOBP1 of *An. gambiae* (Wogulis et al., 2006), and *C. quinquefasciatus* CquiOBP1 (Leal et al., 2008; Manoharan et al., 2014), changes of pH have similarly been suggested to induce rupture of the double hydrogen/ionic bond located at the C-terminus, resulting in the disruption of the binding site and the release of the ligand. In mosquitoes, OBPs are involved in the binding of diverse odorants (Deng et al., 2013; Murphy et al., 2013), including pheromones (Li et al., 2008; Mao et al., 2010) and oviposition attractants (Pelletier et al., 2010a). For instance, in *Ae. aegypti*, OBP1 binds to the insect repellent DEET (Leal and Leal, 2015), and OBP22 binds to fatty acids (Wang et al., 2020), while in *An. gambiae*, OBP1 is involved in the recognition of indole (Biessmann et al., 2010). Further structural and functional studies are required to help understanding better the role of these proteins in the molecular adaptations of the olfactory system in mosquitoes.

### 3.2.2 CSPs

Similar to OBPs, CSPs are small soluble binding proteins (ca. 130 residues in length), possessing an α-helical structure (Angeli et al., 1999), which have been reported in several orders of insects (Nomura et al., 1992; Bohbot et al., 1998; Angeli et al., 1999). Chemosensory proteins are classified as binding proteins, as CSPs possess a hydrophobic binding cavity, yet differ from OBPs as CSPs have a set of only four well-conserved cysteines forming two disulfide bridges between neighbouring residues (Angeli et al., 1999; Picimbon et al., 2000; Sánchez-Gracia et al., 2009). Several, but not all, members of the CSP family are highly expressed in the sensillum lymph and exhibit binding activity towards odorants and pheromones (Leal, 2004; Biessmann et al., 2005; Pelosi et al., 2006; Sánchez-Gracia et al., 2009). The broad distribution of CSPs in insect tissues, especially legs, as well as their structure and temporal expression patterns, suggests that these ubiquitous proteins might have a broader function than OBPs, possibly as molecule carriers involved in the transport of contact chemosensory molecules (Picimbon, 2003; Tegoni et al., 2004; Leal et al., 2013) Chemosensory protein sequences are highly divergent among insect species, *e.g.* only one ortholog has been described between the mosquito *An. gambiae* and *D. melanogaster*, suggesting that CSPs are likely involved in the transport of chemicals exhibiting distinct ligand-binding properties (Picimbon et al., 2000; Picimbon, 2003). Although CSPs are considered binding proteins, the functional role of these proteins in mosquitoes as well as in other insects remains poorly understood.

In *Drosophila*, CSPs form a single conserved class of binding proteins consisting of only four members (Sánchez-Gracia et al., 2009). In mosquitoes, an average of seven CSPs were identified from 19 different *Anopheles* genomes, 27 CSPs in *Cx. quinquefasciatus*, 43 in *Ae. aegypti*, and 83 in *Ae. albopictus* (Mei et al., 2018). Most functional studies on CSPs to putative ligands, have been performed with proteins expressed in heterologous systems through various methods, the most recent being fluorescent binding assays, which reveal that CSPs bind different compounds depending on insect species (Pelosi et al., 2006). Generally, CSPs are found to be broadly tuned (Ban et al., 2002, 2003), although in some cases, these proteins can be more narrowly tuned to specific classes of compounds (Briand et al., 2002; Calvello et al., 2003; Ozaki et al., 2005). Similar to OBPs, CSPs are suggested to be involved in the transport of hydrophobic

compounds, *e.g.* pheromones, odorants, or fatty acids, to their receptor (Lartigue et al., 2002). In insects, even though CSPs are believed to be involved in salient odorant recognition and translocation of volatile chemicals to the ORN dendritic membrane (Pelosi et al., 2006; Sánchez-Gracia et al., 2009), there is still no clear evidence that CSPs participate directly in olfaction or gustation.

## 3.3 Chemosensory receptors

In insects, the molecular mechanisms involved in the detection of odorant molecules require diverse members of the three main gene families; ORs, gustatory receptors (GRs) and ionotropic receptors (IRs). These chemosensory receptors are expressed on the dendritic membrane of the ORNs (Clyne et al., 2000; Neuhaus et al., 2005; Wilson and Mainen, 2006; Benton et al., 2009). In mosquitoes, alike in other insect species (Liu et al., 2014; Latorre-Estivalis et al., 2017), the chemosensory receptors, ORs and IRs, have been shown to be primarily expressed in the main olfactory organs, *i.e.* antennae and maxillary palps (Leal et al., 2013; Matthews et al., 2016b; Lombardo et al., 2017). In contrast, GRs are predominantly detectable in the rostrum, maxillary palp and proboscis (Zhou et al., 2014; Matthews et al., 2016a; Lombardo et al., 2017), as well as labella and tarsi where all the sugar receptors were detected in *Ae. aegypti* (Sparks et al., 2013b), which coincides with their conserved role in taste (Kent et al., 2008). In the case of ORs and IRs, the detection of odorant volatiles has been demonstrated to be mediated by the association between these receptors and highly conserved co-receptors (Vosshall et al., 2000; Robertson et al., 2003a; Hallem et al., 2004; Liu et al., 2010; Abuin et al., 2011; Hussain et al., 2016; Jason Pitts et al., 2017), with which these form heteromeric complexes (Larsson et al., 2004; Neuhaus et al., 2005; Abuin et al., 2011). Their structure and role are further discussed. In insects, the sensory neuron membrane proteins (SNMPs), expressed in subpopulations of ORNs, have been shown to be involved in the detection of pheromones (Benton et al., 2007; Vogt et al., 2009) and other odorants (Bohbot and Vogt, 2005; Pelletier and Leal, 2011). The last step of the olfactory process, *i.e.* the termination and degradation of the chemical

signals, is governed by odorant degrading enzymes (ODEs) (Ishida and Leal, 2005, 2008; Che-Mendoza et al., 2009; Durand et al., 2011; Gong et al., 2013; Edi et al., 2014; Li et al., 2015; Ishak et al., 2016).

### 3.3.1 ORs

A major breakthrough in olfactory research was the identification of a large multigene family of seven transmembrane proteins, members of the superfamily of G protein-coupled receptors (GPCRs), encoding olfactory receptors in mammals (Buck and Axel, 1991). The use of a computer algorithm targeting specific structural properties of proteins, rather than particular sequences, in the *Drosophila* genome databases, resulted, a few years later, in the description of a novel gene gamily fulfilling the criteria of *ORs* (Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999). The insect OR genes encode a family of seven-transmembrane domain receptor proteins of approximately 400 amino acid residues in length that are expressed in the dendrites of ORNs (Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999; Elmore and Smith, 2001; Barnea et al., 2004), and can detect and discriminate volatile hydrophobic molecules of less than ca. 300 Daltons in molecular weight (Wilson and Mainen, 2006). Insect ORs, present an inverted membrane topology, which differs from that of GPCRs, as the short N-terminal domain is located intracellularly and their C-terminus is extracellular (Vosshall et al., 1999; Benton et al., 2006). In insects, the sense of olfaction has been demonstrated to be mediated by the association between these ORs, and a highly conserved co-receptor, named ORco (Vosshall et al., 2000; Robertson et al., 2003a; Hallem et al., 2004). Within ORNs, ORs can form heteromeric complexes between this atypical OR, Orco, and conventional tuning ORs (Larsson et al., 2004; Neuhaus et al., 2005).

Genomic and expression analyses have identified a total of 79 and 88 ORs in the mosquito species *An. gambiae* and *Ae. aegypti*, respectively (Hill et al., 2002; Bohbot et al., 2007a), and 62 ORs in *D. melanogaster* (Robertson et al., 2003a). In insects, ORs are the most prevalent chemosensory-related gene family in olfactory tissues (Pitts et al., 2011; Leal et al., 2013; Rinker et al., 2013a, 2013b; Matthews et al., 2016b;

Lombardo et al., 2017; Tallon et al., 2019), which reveals their implication in the modulation of key odour-mediated behaviours. Moreover, Orco is the most abundant ORs identified in the mosquito antennae (Pitts et al., 2011; Rinker et al., 2013b; Matthews et al., 2016b; Lombardo et al., 2017; Tallon et al., 2019), like described in other insect species (Latorre-Estivalis et al., 2017). As a gene family, insect ORs are divergent, as most ORs exhibit a relatively low level of identity to each other (ca. 20%) at both the inter- and intra-specific levels (Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999). In *Drosophila*, OR sequences exhibit the strongest homology within their C-terminal domain (Clyne et al., 1999; Benton et al., 2006). In contrast, the most divergent region in the sequences of *Drosophila* ORs corresponds to a stretch of 30 amino acid residues located at the first extracellular loop and along the third transmembrane domain (Clyne et al., 1999). Structural changes occurring in these highly divergent regions have previously been shown to be involved in the modulation of the activation of odorant-tuning receptor-dependent channels, therefore affecting OR-ligand sensitivity (Xu and Leal, 2013; Hughes et al., 2014; Turner et al., 2014), in which conformational changes in the protein structure, due to even minor changes in amino acid sequences of ORs, lead to variation in both the sensitivity and specificity of a receptor to its ligand(s) (Curran and Engelman, 2003; Hill et al., 2015).

The species-specific *OR* expansions described between *Drosophila* and mosquitoes may reflect distinct host preferences between species, *e.g.* the detection of human odours in mosquitoes (Hill et al., 2002). Yet, of note, Orco is highly conserved between *Drosophila* and mosquitoes (ca. 80% amino acid identity) (Hill et al., 2002; Pitts et al., 2004; Bohbot et al., 2010), exhibiting exceptional sequence and functional conservation through approximately 250 million years of evolution, which reveals a crucial function in insect olfaction (Jones et al., 2005). However, aside Orco, considerable divergence has been described among OR sequences, both between *Ae. aegypti* and *An. gambiae* (Bohbot et al., 2007a) and among anophelinae lineages (Neafsey et al., 2015). While the conservation of a subpopulation of ORs between mosquito species might indicate shared ecological constraints, the high degree of divergence between *ORs* has been suggested to likely reflect distinct needs between these species, *e.g.* different degrees of anthropophily and ecological niches (Bohbot et al., 2007a).

The initial steps in odour detection require the binding of a ligand to the OR, resulting in the rapid synthesis of the second messenger cyclic adenosine monophosphate (cAMP) through a G protein (Kaupp, 2010). The synthesis of cAMP is involved in the opening of ion-gated channels through the activation of Orco (Wicher et al., 2008). As the structure of Orco has been shown to function as an ion channel (Butterwick et al., 2018), the aforementioned second messenger pathway might be affected. The heteromerisation between the ligand-selective ORs, which confer the odour specificity, and the ubiquitous Orco within the ORN dendritic membranes, is a crucial step in the detection of odorant molecules, which strongly enhances the functionality of these receptors (Dobritsa et al., 2003; Larsson et al., 2004; Neuhaus et al., 2005; Benton et al., 2006; Sato et al., 2008; Stengl and Funk, 2013). In mosquitoes, Orco has been shown to be required to promote functional reconstitution of odour-evoked signalling in ORNs, in which the conventional tuning ORs do not respond to odorants when expressed individually without Orco in both mosquitoes (DeGennaro et al., 2013; Liu et al., 2016), and *Drosophila* (Larsson et al., 2004; Benton et al., 2006; Ditzen et al., 2008). Interestingly, in the absence of $CO_2$, ORco-mutant female *Ae. aegypti* and *An. gambiae* have been shown to no longer be attracted to human odours, and in the presence of $CO_2$, females cannot discriminate between human and non-human hosts (DeGennaro et al., 2013). Thus, in insects, the olfactory mode of action of odour detection requires the association of tuning ORs and Orco.

### 3.3.2 GRs

Further genome mining identified a distinct large family of membrane proteins, which were named GRs due to their spatial expression patterns in gustatory tissues (Clyne et al., 2000; Dunipace et al., 2001; Scott et al., 2001). In insects, these receptors are mainly expressed in gustatory receptor neurons (GRNs) housed in chemosensory sensilla distributed on gustatory organs, *e.g.* proboscis, wing margins, as well as the maxillary palps, genitalia and tarsal segments of the legs (Clyne et al., 2000; Dunipace et al., 2001; Scott et al., 2001; Kent et al., 2008), which coincides with their potential role in the detection of tastants (Scott et al., 2001; Freeman et al., 2014) and CO2 (Jones et al., 2007; Kwon et al., 2007; Erdelyan et al., 2012a; McMeniman

et al., 2014). Insect GRs are a family of transmembrane receptors, phylogenetically related to ORs, consisting of seven predicted transmembrane domains, with an overall sequence length ca. 380 amino acid residues, similar to that of the ORs (Clyne et al., 2000; Dunipace et al., 2001). The conserved positions of a certain number of introns suggest that the family possess a common ancestral gene (Clyne et al., 2000; Robertson et al., 2003a). Gustatory receptors present a higher sequence conservation, except in the regions of the C-terminal, which contains a conserved motif (Clyne et al., 2000; Robertson et al., 2003b). Orthologous GRs have been described between *Drosophila* and mosquitoes, *e.g.* the $CO_2$ detecting GR (Jones et al., 2007; Kwon et al., 2007; Robertson and Kent, 2009). Gustatory receptors, which are structurally-related to ORs, possess a high degree of sequence divergence among insect species, suggesting that GRs may be involved in multiple different functions.

While a total of 68 *GRs* were identified in *Drosophila* genome (Robertson et al., 2003a), 76 and 91 *GRs* were described in *An. gambiae* (Hill et al., 2002) and *Ae. aegypti* (Kent et al., 2008), respectively. Two GRs, GR1 and GR3, have been demonstrated to be involved in the detection of $CO_2$ in mosquitoes (Jones et al., 2007; Lu et al., 2007; Erdelyan et al., 2012a; McMeniman et al., 2014), whereas the role of GR2 remains unknown. Moreover, *Drosophila* GRs can play other roles in addition to the detection of $CO_2$, *e.g. GR68a, GR32* and *GR39a* act as pheromone receptors and are involved in courtship behaviour in *Drosophila* (Bray and Amrein, 2003; Miyamoto and Amrein, 2008; Watanabe and Toba, 2011), and the homologues of the *Drosophila GR28* gene are involved in both taste (Mishra et al., 2018) and thermosensation (Ni et al., 2013). However, the functional role of mosquito GRs remain unknown.

### 3.3.3 IRs

Insect IRs are phylogenetically unrelated to ORs or GRs, and represent an ancient family of ligand-gated ion channels derived from ionotropic glutamate receptors (iGluRs) (Littleton and Ganetzky, 2000; Benton et al., 2009; Croset et al., 2010). Two distinct subfamilies of IRs have been identified: the conserved "antennal IRs" described as olfactory receptors in all insects studied, and the species-specific "divergent IRs" (Croset et al., 2010). Ionotropic receptors have been shown to be mainly expressed in

dendrites of chemosensory neurons that do not express ORs (Benton et al., 2009; Croset et al., 2010), although both IR76b and OR35a/ORco have been shown to be expressed in the same subset of ORNs in *Drosophila* (Couto et al., 2005; Yao et al., 2005). Ionotropic receptors have a relatively short extracellular N-terminus preceding two ligand-binding domains, whose two lobes (S1 and S2), and a short intracellular C-terminus (Benton et al., 2009; Croset et al., 2010). The "antennal IRs" express two or three IR genes, in addition to one or both of the broadly expressed co-receptors (Benton et al., 2009; Abuin et al., 2011). Three broadly expressed IR co-receptors, the IRcos (IR8a, IR25a and IR76b) have been described (Benton et al., 2009), and are the most abundant IRs identified in the antennae of mosquitoes (Pitts et al., 2011; Rinker et al., 2013b; Matthews et al., 2016b; Lombardo et al., 2017; Tallon et al., 2019), and other insect species (Latorre-Estivalis et al., 2017). These IRcos have been suggested to act in concert with the others tuning IRs (Abuin et al., 2011). Of note, IR25a is the IR with the most conserved primary sequences compared to iGluRs, revealing that this IR may be the most similar to the common ancestral IR (Croset et al., 2010).

A total of 61 *IRs* have been described in *Drosophila*, 15 of which are "antennal *IRs*" (Benton et al., 2009). In mosquitoes, 46, 95, and 69 putative IR genes have been identified in *An. gambiae*, *Ae. aegypti*, and *C. quinquefasciatus*, respectively (Croset et al., 2010; Chen et al., 2017). In insects, the heteromerization of one of the IRco with a conventional IR, similar to ORs, has been shown to be necessary and sufficient for the detection of environmental volatile chemicals and mediation of odor-evoked responses (Liu et al., 2010; Abuin et al., 2011; Hussain et al., 2016; Jason Pitts et al., 2017). In mosquitoes, IRs act as sensors of chemical cues, including biogenic amines and neuropeptides previously demonstrated to be involved in the modulation of odor responses (Siju, 2009), *e.g.* the complex of IR41a with one of the two Ircos, IR25a or IR76b, is sensitive to amine/imine compounds in *An. gambiae* (Jason Pitts et al., 2017).

Ionotropic receptors have been demonstrated to specifically respond to amines or acid-based volatiles and other odorants that are not detected by ORs (Hallem and Carlson, 2006; Silbering et al., 2011; Hussain et al., 2016). In addition, receptors belonging to the *An. gambiae* IR75k complex were shown to specifically respond to carboxylic acids (Jason Pitts et al., 2017), which are semiochemicals that have been implicated in mediating host-seeking by adult females, *i.e.* being major components of human sweat (Cork

and Park, 1996b; Bernier et al., 2000). Besides their function in olfaction, IRs have been found to play a potential role in thermosensing in *Drosophila* (Ni et al., 2016). Despite the potential diverse roles of IRs in insect chemosensation, future functional characterization remains necessary to elucidate the implications of IRs in the detection of biologically relevant odorants.

### 3.3.4 SNMPs

In insects, SNMPs are speculated to be involved in pheromone detection (Rogers et al., 2001; Benton et al., 2007; Jin et al., 2008; Vogt et al., 2009). These insect membrane proteins (ca. 520 residues in length) belong to the CD36 family of proteins (Acton et al., 1994; Ohgami et al., 2001), and are expressed in the receptive membranes of the ORN dendrites (Rogers et al., 2001; Benton et al., 2007; Jin et al., 2008). The SNMPs consist of a large extracellular loop containing nine cysteines anchored by two transmembrane domains, out of which at least six are involved in disulfide bonds (Rasmussen et al., 1998). While the first SNMP, SNMP1, was discovered in the moth species *Antheraea polyphemus* (Rogers et al., 1997), a second SNMP, SNMP2 was later identified in *M. sexta* (Rogers et al., 2001). Later studies have found that these proteins are highly conserved among insects, particularly in holometabolous species (Nichols and Vogt, 2008; Vogt et al., 2009). In this membrane-bound protein family, SNMP1 has been demonstrated to be involved in pheromone detection in insects (Benton et al., 2007; Vogt et al., 2009). In *Drosophila* and moths SNMP1 is involved in pheromone detection, (Rogers et al., 2001; Benton et al., 2007; Jin et al., 2008; Pregitzer et al., 2014), whereas SNMP2 is speculated to be involved in the transport of long-chain fatty acids and interactions with other proteins (Silverstein and Febbraio, 2009). The two types of SNMPs were suggested to play key roles in odorant reception (Bohbot and Vogt, 2005; Pelletier and Leal, 2011), however their functional role remains to be elucidated.

### 3.3.5 ODEs

Odorants and pheromones are in part noxious chemicals potentially toxic for insects, and their persistence in the perireceptor environment may negatively impacts the temporal coding of the chemical signal (Pelosi, 1996). Thus, the final step in the olfactory process, corresponding to the signal termination, is required to control the presence of odorants in the perireceptor space and ensure these molecules do not impede the sensitivity of the ORNs. For this purpose, a catalogue of ODEs is present in the sensillum lymph. Two possible pathways for ODE function have been proposed: i. ODEs may participate in the rapid degradation of the stimulus leading to signal inactivation (Ishida and Leal, 2005, 2008), or ii. ODEs may function as a molecular trap to degrade the signals, similar to what has been suggested for OBP2 in *Ae. aegypti* as its ligand may not be ejected via pH-dependent conformational changes. In mosquitoes, detoxifying enzymes, in particular, cytochrome P450s and glutathione-S-transferase, likely act as ODEs, have received considerable attention due to their potential role in insecticide resistance (Che-Mendoza et al., 2009; Gong et al., 2013; Edi et al., 2014; Li et al., 2015; Ishak et al., 2016). In the context of insect control, ODEs have been suggested to represent potential targets in the disruption of odorant perception (Natale et al., 2003; Vogt, 2003; Riffell et al., 2009; Nikbakhtzadeh et al., 2014). The exposure of haematophagous insects to repellents has been shown to alter the expression of these enzymes, particularly of cytochrome P450s (Jaramillo Ramirez et al., 2012; Koloski et al., 2019). However, the exact roles of ODEs and the mechanism(s) underlying the interactions of these enzymes with the odorants remains unclear.

## 3.4 Non-canonical chemosensory receptors

Two families of other membrane bound proteins contain non-canonical chemosensory receptors, transient receptor potential (TRP), and pickpocket (PPK) ion channels, are conserved in all animals (Ramsey et al., 2006; Venkatachalam and Montell, 2007; Ben-Shahar, 2011; Zelle et al., 2013). While TRP channels are broadly represented in various tissues, PPK are

mostly abundant in peripheral tissues involved in contact chemosensation, *i.e.* legs, proboscis (Matthews et al., 2016b; Lombardo et al., 2017). Transient receptor potential ion channels function as pore-forming subunits constituted of a calcium ion-permeable channel (Vaca et al., 1994; Xu et al., 1997; Liu et al., 2007a). In contrast, PPKs act as non voltage-gated, amiloride-sensitive sodium channels (Garty and Palmer, 1997; Bianchi and Driscoll, 2002). Both families are involved in a wide number of sensory modalities, including *e.g.,* chemosensation, mechanosensation, thermosensation and hygrosensation (O'Hagan et al., 2005; Lin et al., 2005; Liu et al., 2007b; Wang et al., 2009; Zhong et al., 2010; Kwon et al., 2010; Árnadóttir et al., 2011; Maekawa et al., 2011; Lu et al., 2012; Starostina et al., 2012; Toda et al., 2012; Tsubouchi et al., 2012; Johnson and Carder, 2012; Zelle et al., 2013; Fowler and Montell, 2013; Guo et al., 2014; Liman et al., 2014; Corfas and Vosshall, 2015; Freeman and Dahanukar, 2015).

### 3.4.1 TRPs

The superfamily of TRP ion channels consists of pore-forming subunits constituted of a calcium ion-permeable channels (Ramsey et al., 2006; Venkatachalam and Montell, 2007), which can be activated by a variety of different stimuli through various mechanisms (Vaca et al., 1994; Xu et al., 1997; Liu et al., 2007a; Venkatachalam and Montell, 2007; Fowler and Montell, 2013). In insects, TRPs participate in diverse sensory modalities, including heat, light, mechanosensation and chemosensation (Liu et al., 2007b; Wang et al., 2009; Kwon et al., 2010; Maekawa et al., 2011; Fowler and Montell, 2013; Corfas and Vosshall, 2015). In mosquitoes, similar to other insects (Venkatachalam and Montell, 2007; Fowler and Montell, 2013), TRPs possess a seven transmembrane structure, and generally exhibit a broad expression in various tissues, which may reflect this wide diversity of potential functions (Matthews et al., 2016b; Tallon et al., 2019). A total of 15 TRPs has been identified in *Ae. Aegypti* (Matthews et al., 2016b). In insects, TRPs play a role in both olfaction and gustation (Al-Anzi et al., 2006; Kang et al., 2010; Kim et al., 2010; Kwon et al., 2010). For instance, in *D. melanogaster*, TRPA1 is necessary to trigger aversive behaviour to the insect repellent compound, citronellal (Kwon et al., 2010). Moreover, both TRPA1 and Pain are expressed in GRNs in mouthparts, and can be activated by aristolochic acid (Kim et al., 2010), and small irritant chemicals (Al-Anzi et al., 2006; Kang et al., 2010). Since orthologs have been described between

all TRPs identified in *Drosophila* and *Ae. aegypti* (Fowler and Montell, 2013; Matthews et al., 2016b), the implication of *Drosophila* TRPs in the detection of insect repellents, encourages further functional characterisation of mosquito TRPs, which may lead to identify and develop new classes of repellents in the fight against insect-borne disease.

Despite the role of TRPs in olfactory, these proteins are also involved in thermosensation. As insects are poikilothermic, the perception of the slightest changes in environmental temperature is therefore essential, especially in the case of mosquitoes as heat is one of the main factor promoting host-seeking (McMeniman et al., 2014; Cardé, 2015; Zermoglio et al., 2017). Several studies have reported a potential role of TRPs in mosquito thermosensation (Wang et al., 2009; Bohbot et al., 2014; Corfas and Vosshall, 2015; Li et al., 2019). Of particular interest, TRPA1, has previously been demonstrated to function as a heat-sensor in host-seeking female *An. gambiae* (Wang et al., 2009) and *Ae. aegypti* (Corfas and Vosshall, 2015), revealing the critical role of TRPs in the mosquito host-seeking. Moreover, TRPA1 presents a lower temperature activation threshold in *C. pipiens*, which inhabits temperate areas, compared to the mosquito species, *Ae. aegypti* and *An. gambiae*, living in tropical and subtropical regions (Li et al., 2019), revealing a correlation between the functional properties of this TRP and the thermal niche preference of the species. Similarly in *Drosophila*, three TRPs, TRPA1, Pain and Pyrexia (Pyx), are involved in thermosensation, *e.g.* the avoidance response of noxious heat (Neely et al., 2011), and warm thermotaxis (Kwon et al., 2008; Wang et al., 2013).

In mosquitoes, moisture, like temperature, plays an essential role in the detection of vertebrate hosts (McMeniman et al., 2014; Cardé, 2015), and discrimination between individual human hosts (Smart and Brown, 1956; Gilbert et al., 1966). Aside their role in thermosensation, TRPs act as mechanosensors and hygrosensors in *Drosophila* (Walker et al., 2000; Liu et al., 2007b; Sun et al., 2009), *e.g.* the TRPN channel NOMPC is required for touch sensing (Walker et al., 2000), the two TRPA channels, Pain and Pyx, are involved in gravitaxis (Sun et al., 2009), and the TRPV channel, Nanchung (Nan), and TRPA channel, Waterwitch (Wtrw), are necessary for the detection of moist and dry air, respectively (Liu et al., 2007b). Yet, the role of TRPs in hygrosensation in mosquitoes remains unknown. Combined, these studies highlight the implication of these ion channels in mosquito

thermosensation and open the door to applying these findings to the design of improved mosquito control strategies.

### 3.4.2 PPKs

Pickpoket ion channels belong to the family of amiloride-sensitive degenerin/epithelial sodium channels (DEG/ ENaC) (Zelle et al., 2013). All family members share a highly conserved topology including two transmembrane helixes, two short intracellular domains, and a large cysteine-rich extracellular loop (Ben-Shahar, 2011; Zelle et al., 2013). The general physiological functions of these channels remain elusive, even though some members of this family were shown to act as non-voltage-gated, amiloride-sensitive sodium channels (Garty and Palmer, 1997; Bianchi and Driscoll, 2002). The broad expression patterns described across various tissues in both mosquitoes (Bohbot et al., 2014; Sparks et al., 2014; Matthews et al., 2016a), and *Drosophila* (Zelle et al., 2013), suggest that PPKs are involved in various physiological functions. A total of 37 PPKs have been described in *Ae. aegypti* (Matthews et al., 2016b), of which none has been functionally characterised in mosquitoes.

Similar, to TRPs, PPKs transduct the signals involved in chemosensation (Lin et al., 2005; Lu et al., 2012; Starostina et al., 2012; Toda et al., 2012). For instance, DEG/ENaCs is involved in pheromone sensing during courtship in *Drosophila* (Lin et al., 2005; Lu et al., 2012; Starostina et al., 2012; Toda et al., 2012). Furthermore, PPKs have potential roles in insect gustation (Liman et al., 2014; Freeman and Dahanukar, 2015), *e.g.* the activation of PPK28 by low osmolarity was shown to be required for *Drosophila* gustatory water sensation (Cameron et al., 2010; Chen et al., 2010). Interestingly, the *Drosophila* PPK11 and PPK19, expressed in the larval taste-sensing terminal organ and on the taste bristles of the labelum, the legs, and the wing margins of adults, was demonstrated to be involved in the detection of salt concentrations (Liu et al., 2003; Alves et al., 2014).

In mosquitoes, mechanosensation and hygrosensation are important parameters involved in the modulation of odour-mediated behaviours (Takken, 1991; Cardé, 2015). Besides chemosensation, PPKs have also been shown to play a role in mechanosensation (O'Hagan et al.,

2005; Zhong et al., 2010; Árnadóttir et al., 2011; Tsubouchi et al., 2012; Guo et al., 2014), and hygrosensation (Matthews et al., 2019), *e.g.* in *Ae. aegypti*, the DEG/ENaC channel PPK301 was demonstrated to be involved in the control of egg-laying initiation (Matthews et al., 2019). A better understanding how mosquitoes interact with water and therefore oviposition behaviour, which is directly linked to their vectorial capacity, is essential for the development of efficient mosquito control, and other disease vectors of public health. A strong sexual dimorphism in the expression of PPKs was shown in the antennae of *Ae. aegypti*, *i.e.* among all the PPKs detected, only one, PPK00926, was found in the antennae of both males and females (Tallon et al., 2019). Although no PPK has been functionally characterized in mosquitoes yet, PPK00926 belong to the subfamily V described as mechanosensors (Zelle et al., 2013). Moreover, the lower abundance of PPKs, together with a decrease of TRP transcript abundances, was speculated to be linked to the reduced sensitivity to both chemical and mechanical stimuli in the maxillary palp of female *Ae. aegypti* observed after a blood meal (Hill et al., 2019). Thus, members of the family DEG/ ENaCs should not be overlooked but rather be considered as further candidate receptors underlying the detection of various categories of signals in insects.

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# 4. Molecular basis of olfaction

## 4.1 Peripheral coding of odorant signals

Over the past two decades, several molecular studies have contributed to a better knowledge of the anatomical and functional organization of insect olfactory system, as well as the molecular and neural mechanisms underlying the detection and processing of olfactory signals, *i.e.* how insects can discriminate among thousands of odorants and the myriads of odorant combinations (Vosshall et al., 1999, 2000; Couto et al., 2005; Fishilevich and Vosshall, 2005; Goldman et al., 2005). However, a lot remains to be investigated, *e.g.* how many odorant stimuli are perceived and discriminated amongst, and how odorant molecules are perceived and processed. In insects, the sophisticated olfactory system can be analysed at different levels, starting with reception of semiochemicals at the periphery (which is the focus of this chapter), their integration in higher processing centres and ultimately, the translation into behavioural ouput (which is beyond the scope if this thesis) (Malnic et al., 1999; Leal, 2013). In line with the available literature, the present chapter is biased toward ORs and IRs.

### 4.1.1 Detecting and encoding odours

Insects constantly manoeuvre through a considerable amalgam of active components, and have the ability to discriminate between appropriate blends and ratios (Bruce et al., 2005). Moreover, in nature, the modulation of odour-mediated behaviours often relies on the discrimination of biologically active

kairomones, within a multitude of volatiles emitted at different ratios, rather than only on the presence or absence of particular odorants. The stereochemical theory, based on the hypothesis that odorant detection and discrimination can only occur when the structure of an odorant molecule matches the structure of its binding site, remains the dominant theory in the research field of insect olfaction (Amoore, 1963; Amoore et al., 1964). The identity of an olfactory stimulus relies on the intensity and quality of the signal, as well as on both spatial and temporal profile of the odour stimulus, *i.e.* how the information is encoded by the ORNs.

The detection of the olfactory stimulus specificity depends on the identity of odorant molecules, which may alter the sentivitity of the ORNs and its signal. While certain ORs, *i.e.* narrowly tuned ORs considered as specialist channels, are capable of detecting one or a few ligands with high specificity, ligand-OR interactions can also be relatively non-specific (Carey et al., 2010; Wang et al., 2010). The identity of odorants has been suggested to be encoded in the activity of multiple ORNs (De Bruyne and Baker, 2008), which is in line with the fact that most ORNs respond to subsets of odorants, and several odorants can trigger responses from several ORNs (Carey et al., 2010; Wang et al., 2010). One can ask whether structurally similar odorant molecules are able to trigger similar ligand-ORs responses in insects. Based on their structural resemblance, octenol analogs have been suggested to be detected by specific receptors, while compounds sharing little similarity in their chemical structure tend to exert antagonist effects (Bohbot and Dickens, 2012). For instance, the compound 2-undecanone is able, alone, to selectively activate OR8 (Bohbot and Dickens, 2010), *i.e.* an OR previously shown to be tuned to octenol (Bohbot and Dickens, 2009; Grant and Dickens, 2011). The intensity of the signal, *i.e.* odorant concentrations, also plays a considerable role in the OR-ligand interactions (Malnic et al., 1999; Hallem and Carlson, 2006; De Bruyne and Baker, 2008; Wang et al., 2010). In *D. melanogaster*, a general decrease in odorant concentrations was associated with a decrease in the number of ORs tuned, as well as in the population of responsive neurons, while the re-augmentation in the concentration of odorants resulted in the recruitment of additional ORs and changes in the combination of receptors involved in the recognition of the ligand (Malnic et al., 1999). Moreover, broadly-tuned ORs were demonstrated to be more narrowly tuned when potential ligands were delivered at low concentrations (Hallem and Carlson, 2006; Wang et al., 2010).

In insect olfactory system, a marked spatial compartmentalization of ORNs into the sensilla has extensively been described in sensory organs (Bruyne et al., 2001; Lu et al., 2007; Kaissling, 2009). In *D. melanogaster*, ORs expressed in different ORNs of similar types of sensilla have been found to be unrelated in terms of *OR* sequences (Couto et al., 2005). Functional and phylogenetic analysis of ORs revealed that receptors encoded by highly-related genes, *i.e.* presenting analogous sequences, tend to be tuned to similar types of compounds in both *Drosophila* (Hallem et al., 2004; Bruyne et al., 2010) and lepidopteran (de Fouchier et al., 2017). Functional orthology has also been demonstrated among various species of mosquitoes (Lu et al., 2007; Bohbot and Dickens, 2009; Pelletier et al., 2010b). For instance, the mosquito OR subfamily including OR2 and OR10, which is tuned to indole in *An. gambiae* (Carey et al., 2010; Wang et al., 2010), *Cx. quinquefasciatus* (Pelletier et al., 2010b), and *Ae. aegypti* (Bohbot et al., 2011a), was shown to be one of the most conserved ORs identified within these three species (Pelletier et al., 2010b; Bohbot et al., 2011a). Moreover, ORNs that share the same space, have been described as influencing their neighbouring ORNs' responsiveness (Andersson et al., 2010; Su et al., 2012), *e.g.* in *An. gambiae*, the activation of the cpB neuron by 1-octen-3-ol results in the inhibition of its partner neuron, cpA, which is sensitive to $CO_2$ (Su et al., 2012). The aforementioned spatial profile of odour coding has been suggested to warrant both a broad detection of odorant molecules at the periphery, *i.e.* the presence, in the same sensilla, of ORs that are unrelated in terms of *OR* sequences and tuned to different odorants, and a specialized integration centrally as closely-related ORs tend to map closely-positioned glomeruli (Couto et al., 2005).

Temporal coding of how odorants are represented in the peripheral olfactory system, *i.e.* how odour identity and intensity vary in time leading to variation in the distribution of ORN firing activity in time, is an important part of how the olfactory signal is detected and processed (Laurent, 2005; Carey et al., 2010). Although aldehydes have been shown to elicit similar responses in the same ORNs in *Ae. aegypti*, the temporal characteristics of these responses differ (Chen et al., 2019). Similarly in *C. quinquefasciatus*, the temporal characteristics of the response to indole has been shown to be different between distinct ORNs (Hill et al., 2009). In *Drosophila*, within a single ORN class, a wide variation has been described in the temporal response of ORNs, *e.g.* one ORN exhibit an abrupt

termination of firing shortly after the end of the stimulus while the spikes continue long after the stimulation in the other ORN (Bruyne et al., 2001). This variation in temporal coding has been shown to be linked to the ligand-OR interaction, rather than being dependent on the odorant concentration (Hallem and Carlson, 2006). Combined, these studies have provided good insights into the temporal dynamics of the responses of ORNs to structurally-related compounds, which is essential in understanding better the peripheral coding of the insect olfactory system. Even though, a lot of advances to decipher the insect olfactory code and olfactory pathways have been made, including in mosquitoes (Qiu et al., 2006b; Carey et al., 2010; Wang et al., 2010), and *D. melanogaster* (Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999), as well as other disease vectors (Wigglesworth, 1953; Barrozo et al., 2017), a lot remains to be understood in regards to the modulation of odour coding in insects.

### 4.1.2 Combinatorial coding versus labelled lines

Large-scale heterologous screening studies of the OR repertoires in *An. gambiae* and *D. melanogaster* have revealed considerable differences in OR specificities, *i.e* from extremely narrow to broad tuning (Dobritsa et al., 2003; Hallem et al., 2004; Yao et al., 2005; Hallem and Carlson, 2006; Xia et al., 2008; Carey et al., 2010; Wang et al., 2010; Haverkamp et al., 2018). For instance, ca. 10% of *Drosophila* OR repertoire is estimated to be narrowly tuned to specific cues (Haverkamp et al., 2018), *e.g.* the aggregation and sex pheromone 11-cis-vaccenyl acetate (cVA) is solely recognized by the combination of Or67d with ORco (Kurtovic et al., 2007). Insect OSNs that respond to sex or aggregation pheromone compounds are often highly specific (Leal, 2004; Wanner et al., 2010; Montagné et al., 2012). This high specificity of pheromone detection has been suggested to allow the maintenance of a high fidelity in the mate recognition system, and ensure reproductive success (Leary et al., 2012). Interestingly, in recent years it has become more apparent that non-pheromonal compounds can also be detected by highly specific receptors (Bruce and Pickett, 2011; Hansson and Stensmyr, 2011; Mathew et al., 2013; Liu et al., 2014; Dweck et al., 2015). For instance, a dedicated olfactory pathway was demonstrated in *D.*

*melanogaster*, in which citrus volatiles are detected exclusively via the receptor OR19a expressed in a single class of ORNs (Dweck et al., 2013).

In insects, it was first proposed that individual ORs are expressed in separate non-overlapping populations of ORNs (Vosshall et al., 1999), as the described ratio of expressed genes to neuronal classes coincides with a model in which each individual ORN expresses a small number of ORs (Clyne et al., 1999). In *Drosophila*, in which the tuning OR repertoire is relatively small (~60), most ORNs, except in the case of ORco, express only one tuning OR (Vosshall et al., 1999; Couto et al., 2005). However, two major working hypotheses are still disputed in order to explain receptor tuning in insects. In one hand, the labelled-line coding of odorant stimuli involves narrow tuning ranges through the implication of specialised ORs (Malnic et al., 1999; Walker et al., 2016). On the other hand, the peripheral combinatorial coding embraces the concept of broad tuning ranges involving the association of several receptors either broadly or narrowly tuned in the creation of a combined signal (Malnic et al., 1999; Walker et al., 2016; Haverkamp et al., 2018). In insects, one subtype of ORs is generally expressed in a unique class of ORNs and their axons converge within the same glomerulus, a rule commonly referred to the 'one neuron: one receptor: one glomerulus' paradigm. In comparison to combinatorial coding, labelled line coding has been demonstrated to be required for the necessity of a faster processing of signals and more efficient discrimination of structurally similar molecules, *i.e.* narrowly tuned receptors are particularly essential in the case of sex- and danger-related signals (Haverkamp et al., 2018). While the speed required to assess an odour source, *i.e.* capacity of ORNs to quickly detect odorants, is crucial in insects (Vickers et al., 2001), a rapid response to the pockets of clean air between odorant strands is just as important, and has been shown to be slightly slower for host-odours (Haynes and Baker, 1989), than for pheromones (Baker and Haynes, 1987; Vickers and Baker, 1996). Furthermore, the importance of labeled coding in insect olfactory system in pest management has brilliantly been discussed (Haverkamp et al., 2018), *i.e.* the existence of a direct link between dedicated olfactory pathways and a specific behavior may render the insect particularly vulnerable to odour-based traps, and targeted mutagenesis-induced behavioural disruption (Kistler et al., 2015; Hammond et al., 2016; Dong et al., 2018).

Despite the canonical 'one neuron: one receptor: one glomerulus' rule, there are few examples in which two to three ORs are co-

expressed in a single ORN class (Fishilevich and Vosshall, 2005; Goldman et al., 2005; Koutroumpa et al., 2014; Karner et al., 2015), and one glomerulus can receive convergent input from two distinct subsets of ORNs (Fishilevich and Vosshall, 2005). The concept of combinatorial coding, considered as a "relaxed" adaptation of the aforementioned stereochemical model of olfaction (Rinaldi, 2007), relies on the hypothesis that ORNs are broadly tuned sensors, expressing ORs, either broadly or narrowly tuned, which respond to multiple ligands, and that single ligands can activate several ORs, and consequently multiple glomeruli (Malnic et al., 1999; Andersson et al., 2015) (Bruyne et al., 2001). A large number of odorant molecules is likely to be detected by a repertoire of receptors which are broadly tuned (Malnic et al., 1999; Bushdid et al., 2014), which was suggested to result in a higher degree of flexibility in the olfactory system (Andersson et al., 2015).Similarly to ORs, several subsets of ORNs have been shown to express a combination of IRs responding to various odorants in *Drosophila* (Benton et al., 2009). In general, combinatorial coding has been adopted as the most probable explanation of how insects "encode" their odour environments (Hallem et al., 2004; Hallem and Carlson, 2006; Galizia and Szyszka, 2008; Touhara and Vosshall, 2009). Multiple advantages have been suggested regarding the evolutionary conservation of combinatorial coding in insect olfactory systems, including the possibility to render the olfactory system more robust in response to rapid changes or disturbances in odour perception, *e.g.* modulation of ligand-response profile through co-expression of ORs by involving other receptors responding to the compound in case one has failed (Fishilevich and Vosshall, 2005). However, even though the combinatorial coding scheme can explain how individual ORs can respond to multiple ligands, it does not clarify why different molecules can have similar smell and why similar molecules can have distinct odours. Moreover, the combinatorial coding scheme was described as counterintuitive from an evolutionary point of view, as it implies a continuous exposure to multiple odorants, increasing the risk of overstimulation (Bohbot and Pitts, 2015). In contrast, combinatorial coding has also been suggested to reduce the presence of background noise, which increases the capacity of discriminating salient signals and allows them to be sent forward in higher olfactory centres (Trona et al., 2013).

## 4.2 Regulation of chemosensory gene expression

Although odour-mediated behaviour underlying vectorial capacity of mosquitoes, *i.e.* sugar- and host-seeking, and oviposition site selection, are rather well documented, the molecular mechanisms underlying their modulation remains poorly understood. During the past decade, the advent of next-generation sequencing (NGS) techniques and heterologous gene expression systems, leading to the availability of multiple annotated mosquito genomes (V Nene, JR Wortman, D Lawson, 2007; Arensburger et al., 2010; Matthews et al., 2016b), have contributed to a better understanding of the molecular basis of insect olfaction (REF). Transcriptome analyses, through the use of ribonucleic acid (RNA)-sequencing (seq), together with more accurate genome annotations, have permitted the identification of large gene families involved in the mediation and modulation of these behaviours (Koutsos et al., 2007; Xia et al., 2008; Pitts et al., 2011; Akbari et al., 2013; Zhou et al., 2014; Chen et al., 2015; Matthews et al., 2016b; Latorre-Estivalis et al., 2017, 2020; Lombardo et al., 2017; Tallon et al., 2019). Over time, such studies have provided a comprehensive view of the changes in the expression of protein-coding genes associated with known behavioural shifts in different biological contexts, *i.e.* age, sex, physiological state, infection, and helped identify candidate chemoreceptors of interest for further functional characterization. Systematic efforts to deorphanize these selected chemoreceptors are crucial in order to address the functional relevance of the aforementioned modulation in gene expression (Lu et al., 2007; Bohbot and Dickens, 2009; Carey et al., 2010; Wang et al., 2010; Bohbot et al., 2011b; Scialò et al., 2012; McBride et al., 2014; Omondi et al., 2019; Ruel et al., 2019; Zeng et al., 2019)(Tallon et al. unpublished data). This chapter aims to highlight the different parameters involved in the modulation of the molecular machinery of olfaction in insects, with a special emphasis on mosquitoes.

**Box 5. RNA sequencing**

RNA sequencing (RNA-seq) is a next generation sequencing technique that generates millions of short nucleotide sequences, known as reads, from libraries of short strand cDNA molecules constructed from RNA

extracted from the substrate of interest, *e.g.* mosquito antennae. Since the development of RNA-seq, about a decade ago, it has become a routine research tool which can used to determine a broad range of characteristic of transcription, including gene abundance profile for differential gene expression analysis (DGE), estimation of the frequency of alternative-splicing events and identifying/quantifying transcript sequence variants, *i.e.* single nucleotide polymorphisms (SNPs), and nucleotide insertions and deletions (indels) (Oshlack et al., 2010; Han et al., 2015). The type of cDNA library construction kit used to sequence RNA has to be carefully considered, as this step requires the use of specific adapters, *e.g.* synthetic oligonucleotides of a known sequence, which will ligate to one or both ends of RNA fragments, and produce adapter-ligated cDNA fragments to be later amplified and sequenced. The most common libraries construct cDNA from mRNAs found in total RNA templates, however libraries constructed from other RNA moieties, *e.g.* non-coding and microRNAs, are feasible. Additional enrichment steps, *e.g.* ribosomal RNA depletion, might be required in order to detect low abundance transcripts. Strand-specific libraries maintain the original orientation of the transcripts, which is particularly useful in the case of non-coding RNA.

Once RNA-seq libraries are ready, the selection of additional parameters, including the type and length of reads, *i.e.* the number of base pairs that are read per sequencing cycle, and depth of sequencing, *i.e*. target number of times a specific read should be sequenced, is essential. Single-end (SE) sequencing generates a single read (3′ or 5′) of each cDNA fragment during one cycle, while paired-end (PE) sequencing allows both the forward and reverse fragments to be sequenced. As the distance between each end is known, mapping PE reads is more accurate over repetitive regions, and preferable when a high coverage of a maximum of nucleotides is desired. In contrast, SE sequencing is sufficient when only the count of the reads mapping to a transcript after alignment is necessary, *i.e.* DGE analysis (739). Although, most of the time, SE reads are sufficient to identify the sequenced gene, PE sequencing is preferable for de novo transcript discovery and improves the detection of alternative-splicing (744,745). However, PE sequencing is generally more expensive per base and time-consuming, resulting in a lower read depth for the same experimental cost, and not necessarily required for all types of experiment. Although the length of the sequencing

reads has been shown to not significantly influence DGE analysis, long reads (LR) (> 25-300 bp), when combined to paired-end (PE) sequencing, provide a better overall coverage of the sequencing (739). Moreover, LR facilitate the detection of isoforms in regions that are difficult to sequence through short-read (SR) platforms, *i.e.* highly polymorphic genes (740). However, the detection of individual transcripts by LR platforms heavily depend on the quality of RNA libraries as input, and is usually subject to relatively high error rates, which might decreases the sensitivity of DGE (740). Although it is possible to reduce the high error rates by increasing the read depth, *i.e.* circular consensus sequence consisting in each molecule to be sequenced multiple times (741), this approach is relatively time- and resource-consuming. Thus, while LR can be used in a more qualitative-targeted RNA-seq analysis, SR platforms should be privileged for the detection of low abundance transcripts, or when full-length transcripts are not available, *i.e.* due to RNA degradation (742).

**Box 6. Differential transcript abundance analysis**

Differential gene expression (DGE) analysis is based on statistical comparisons of quantitative changes in expression levels of individual genes and/or transcripts between experimental groups. Differential gene expression analysis is a very useful tool to investigate whether changes in gene and/or transcripts are due to natural random variation. *E.g.* in this thesis, changes in transcript abundances of chemosensory-related genes expressed in the antennae of female *Ae. aegypti*, have been correlated with age, sex and dengue infection.

The analysis begins on receipt of RNA-seq read libraries (see Box 5) in the common FASTQ file format, which includes all the reads in the FASTA format along with base quality scores indicating the quality of the sequences. To begin the analysis, a quality control step is paramount, in order to remove adapter sequences, low-quality reads, uncalled bases and contaminants. At this point, technical errors in sequencing can be checked by combining all the samples into one single 'pooled' sequencing library and multiplying the number of samples by the desired read depth, which will inform the user about the total number of reads expected (742).

Once the sequences are cleaned and the number of expected reads are confirmed, the depth and coverage of sequencing, can be assessed by using the core eukaryotic gene mapping approach (Cegma) (746). A good coverage of sequencing is required for a reliable comparison of quantitative changes in gene expression and/or transcript abundance. The cleaned reads are then mapped to a reference genome or transcriptome, when one is available. This process aligns the nucleotide sequence of the reads with the corresponding sequence in the reference genome or transcriptome. This step necessitates no particular knowledge of the reference genome, and can result in the discovery of new transcripts in need of annotation. When a reference genome or transcriptome is not available, DGE analysis is still possible, through the construction of a transcriptome by the *de novo* assembly of the cleaned reads, *e.g.* assemblage of overlapping reads into longer contiguous sequences (contigs) (747). For both mapping to a reference genome and de novo assembly, the use of short reads (SR, see Box 5) is challenging, as they can sometimes map to multiple locations, and paired end (PE, see Box 5) sequencing and the use of long reads (LR, see Box 5) appears as a good alternative for solving structural rearrangements, *i.e.* gene insertions, deletions or inversions in *de novo* assemblies.

Once the cleaned read libraries are mapped, the quantification of gene expression can begin. Differential gene expression, when assessed using transcriptomics, is generally done by counting the number of aligned reads associated with each transcript, and as such, should be reported as read or transcript abundance, rather than expression. Normalised transcript abundance is calculated by counting the total number of aligned reads and comparing this to the read count and nucleotide length of each gene. Since raw read counts can be affected by the length of the transcript, sequencing depth and library sizes, normalized expression units, such as RPKM (reads per kilo base per million mapped reads), its derivative FPKM (fragments per kilobase of exon model per million reads mapped) and TPM (transcript per million reads mapped) are widely used to estimate expression levels and read counts (738). The identification of a set of differentially expressed genes across experimental conditions via RNA-seq, require the use of count-based methods, including DESeq based on the estimation of fold changes between groups (738,749). The use of cutoffs regarding the threshold of

reliable detection and differential fold change in transcript abundance, remains arbitrary and depends on the biological question and type of samples, *e.g.* in this thesis, the threshold of reliable detection corresponds to 1 TPM, and the differential fold changes were calculated using the beta-binomial Baggerley's test (Baggerly et al., 2003) and false discovery rate-corrected p-values (Benjamini and Hochberg, 1995). In summary, RNA-seq is a large-scale data analyses, considered less biased than microarray-based methods and presenting various advantages in the field of molecular biology, including a high sensitivity for genes expressed at both low and high levels, and low technical variation leading to high levels of reproducibility.

### 4.2.1 Gene expression depends on sex

The chemosensory organs in males and females differ in expression of chemosensory-related genes in many mosquito species, *e.g. Ae. aegypti* (Sparks et al., 2013b; Matthews et al., 2016b; Tallon et al., 2019), *Ae. albopictus* (Chen et al., 2015; Lombardo et al., 2017), *An. quandriannulatus* (Athrey et al., 2020), *An. coluzzii* (Athrey et al., 2020), and *An. gambiae* (Justice et al., 2003; Iatrou and Biessmann, 2008; Pitts et al., 2011), as well as in *D. melanogaster* (Watanabe and Toba, 2011; Shiao et al., 2013; Younus et al., 2014), *C. pomonella* (Anfora et al., 2012), and *R. proxilus* (Latorre-Estivalis et al., 2017, 2020). Several complete transcriptome analysis in mosquitoes have shown a strong sexual dimorphism in transcript abundance (Baker et al., 2011; Matthews et al., 2016b), *e.g.* sex-specific enrichment in chemosensory-related genes expressed in the antennae of *Ae. aegypti* (Matthews et al., 2016b). In mosquitoes, a deeper knowledge of the olfactory repertoire differences between males and females, opens up new possibilities for the development of targets for control strategies.

Among all the chemosensory-related gene families, OBPs consistently exhibit the highest transcript abundances in peripheral neural tissues (Matthews et al., 2016b; Lombardo et al., 2017; Tallon et al., 2019). In addition, OBPs demonstrate the strongest gender-biased expression among the chemosensory-related gene families, and generally display higher transcript abundance in female olfactory tissues compared with males (Justice et al., 2003; Biessmann et al., 2005; Pitts et al., 2011; Lombardo et

al., 2017; Tallon et al., 2019; Athrey et al., 2020). Among the most gender-biased OBPs described in *An. gambiae* and *An. coluzzii*, OBP1 and OBP12, which exhibit enriched expression levels in females (Justice et al., 2003; Biessmann et al., 2005; Athrey et al., 2020), have previously been shown to be involved in the detection of two compounds involved in host-seeking (McBride et al., 2014) and oviposition site selection (Allan and Kline, 1995a), sulcatone (Murphy et al., 2013), and indole (Biessmann et al., 2010), and the mosquito attractants, ammonia and LA (Manoharan et al., 2013), respectively. OBP1 was also described to be more abundant in female *Ae. aegypti* (Matthews et al., 2016b; Tallon et al., 2019). In mosquitoes, similarly to OBPs, ORs present both a higher number and greater transcript abundance in female antennae compared to males (Baker et al., 2011; Pitts et al., 2011; Matthews et al., 2016b; Lombardo et al., 2017; Tallon et al., 2019; Athrey et al., 2020). These results are likely mostly correlated to the greater number of chemosensory trichoid sensilla observed in female antennae (McIver, 1982; Pitts and Zwiebel, 2006). In *Ae. aegypti*, no male-specific ORs were detected, whereas more than half of the ORs identified were female-specific (Tallon et al., 2019). Both OR1 and OR2 are enhanced in female antennae of *An. gambiae* (Iatrou and Biessmann, 2008; Pitts et al., 2011), *Anopheles quadriannulatus* and *An. coluzzii* (Athrey et al., 2020), and respond to 2- and 4-methylphenol, which are components of human sweat (Elissa A.Hallem, A.Nicole Fox, Laurence J.Zwiebel, 2004), as well as to human-derived odorants (Omondi et al., 2019). Similar to the gender-biased transcript abundance and functional characterization of ORs described in females, the modulation of male-enriched OR expression likely reflects the sexually-dimorphic behavioural specialization. For instance, in both *An. coluzzii* and *An. quadriannulatus*, OR27 transcript abundance was found to be considerably more abundant in males compared to females (Athrey et al., 2020), which is interesting as this OR is narrowly tuned to the plant-derived terpenes fenchone and carvone (Wang et al., 2010). The described variation in transcript abundance of OBPs and ORs suggests a potential role of both of these gene families in sex-specific odour-mediated behaviours, and may therefore be promising targets for behavioural disruption.

In mosquito antennae, unlike ORs, there is no major difference in IR and GR abundance between sexes (Pitts et al., 2011; Matthews et al., 2016b; Tallon et al., 2019), although one study has recently shown that the overall expression levels of IRs was considerably higher in

female *An. coluzzii* and *An. quadriannulatus* antennae, compared to males, suggesting a role of IRs in sex-specific modulation of behaviour (Athrey et al., 2020). Moreover, Sparks *et al.* (2013) identified numerous sex-specific expression biases in the labellar and tarsal tissues of *Ae. aegypti*, which may reflect a sensory adaptation of these GRs in sex-specific aspects of mosquito biology (Sparks et al., 2013b). For instance, GR1 was found to be enhanced in female antennae in *An. gambiae*, compared to males (Pitts et al., 2011), and is involved in detection of $CO_2$ (Erdelyan et al., 2012b), which is one of the main volatile cues involved in host-seeking (Gillies, 1980). Similarly, GR39, which is one of the highly male-enriched GRs in *Ae. aegypti* (Sparks et al., 2013b), has a functionally characterized ortholog in *D. melanogaster* involved in maintaining courtship behaviour in males, *e.g.* possibly acting as a chemoreceptor for a non-volatile female sex pheromone (Watanabe and Toba, 2011). Although a relatively balanced transcript abundance is described for antennal IRs and GRs between both sexes, this does not exclude the possibility that these receptors are enriched in ORNs that are part of sex-specific neural circuits underlying sex-related behaviour (Kimura et al., 2005; Lu et al., 2012).

Limited gender-bias expression levels of chemosensory-related genes might reflect common olfactory functions between males and females, as shown by similar sensitivities to the same compounds (Davis, 1977). Similarities in transcript abundance of chemosensory-related genes between sexes may also correspond to universal behaviours, *i.e.* sugar seeking, response to host presence, mate location, between female and male mosquitoes.

### 4.2.2 Gene expression depends on age

In adult mosquitoes, the development of odour-mediated behaviour across ages is correlated with overall age-dependent transcriptional changes in select chemosensory-related genes (Bohbot et al., 2013b; Omondi et al., 2015b, 2019; De et al., 2018; Tallon et al., 2019). The gene abundance of chemosensory-related receptors change start within the 24 first hours following emergence, while less alteration in expression levels is observable around 5-6 days post-emergence (Omondi et al., 2015b, 2019; De et al., 2018; Tallon et al., 2019). A functional specialisation in the antennae and a different rate of accumulation of canonical olfactory receptors between sexes

was speculated to occur in newly-emerged mosquitoes, as little variation has been detected between sexes in maturing individuals (Pitts et al., 2011; Matthews et al., 2016b; Tallon et al., 2019). Recruitment of ORs in the antennae, *i.e.* the expression of additional ORs over time, corresponding with adult age, was described in *Ae. aegypti* (Tallon et al., 2019), suggesting that the sexually-dimorphic increase of transcript abundance is may be linked to different timing in adult maturation between males and females (Koutsos et al., 2007; Tallon et al., 2019). It should be noted, however, that unlike ORs, neither the expression nor the rate of accumulation of IRs were influenced by age in *Ae. aegypti* (Tallon et al., 2019). Several key ORs have been suggested to play a similar role in regulating the onset of host-seeking in *An. coluzzii*, as changes in their sensitivity to cognate human odorant ligands correlate with the age-dependent regulation of host-seeking (Omondi et al., 2019). It has been speculated that the avoidance to two compounds present in human body emanates, 1-hexanol and sulcatone, described in newly-emerged female *An. coluzzii*, is linked to the downregulation in maturing females of OR39 that responds specifically to these compounds (Omondi et al., 2019).

The increase in the transcription level of chemoreceptor genes has also been shown to be linked to the maturation of female mosquitoes for host-seeking (Omondi et al., 2015b, 2019; Tallon et al., 2019). For instance, among all the female-specific ORs described in *Ae. aegypti*, OR103 exhibits higher abundance in host-seeking females at 3-5 days post-emergence (Tallon et al., 2019). This OR is more abundant in the human-preferring subspecies of *Ae. aegypti* (McBride et al., 2014), and is tuned to linalool emitted by human body odour (Tallon et al. unpublished data). Similarly, the increase in expression levels of OR8, as females become capable of host-seeking, is correlated with enhanced sensitivity of its ORN to its ligand, 1-octen-3-ol (Bohbot et al., 2013a). In *An. coluzzii*, both GR22 and OR28, involved in the detection of $CO_2$ (Lu et al., 2007), and multiple vertebrate-associated compounds (Lu et al., 2007; Xia et al., 2008; Carey et al., 2010), respectively, show an increase in transcript abundance between 1 and 4 day post-emergence, with greater transcript abundances in host-seeking females (Omondi et al., 2015b). In *An. coluzzii*, three additional ORs, OR1, OR2 and OR75, responding to human odorants (Elissa A.Hallem, A.Nicole Fox, Laurence J.Zwiebel, 2004; Carey et al., 2010; Bohbot et al., 2011b; Scialò et al., 2012; Omondi et al., 2019; Ruel et al., 2019; Zeng et al., 2019), were

found with higher transcript abundance in host-seeking females (Omondi et al., 2019). A synthetic blend composed solely of the ligands of these three ORs, *i.e.* phenol, butyl acetate and monoterpenes, has been shown to be sufficient to elicit host-seeking behaviour in female *An. coluzzii*, whereas the removal of these ligands rendered the blend unattractive to host-seeking females (Omondi et al., 2019). These studies highlight the importance of describing transcriptional changes occurring at the onset of host-seeking in order to unravel the role of such odorants in host recognition.

### 4.2.3 Physiological state influences gene expression

In female mosquitoes, the olfactory refractoriness of host-seeking, which was demonstrated to occur during the first 48 h following a blood-meal (Klowden and Lea, 1979a; Takken et al., 2001), is not only associated to reduced flight and locomotion activities (Jones, 1981; Klowden, 1981), but also to changes in antennal sensitivity to volatiles (Qiu et al., 2006b, 2013; Siju et al., 2010). For instance, between 24 and 72 h post blood meal, *Ae. aegypti* females display an increased ORN sensitivity to indolic and phenolic compounds, which co-occurs with the drive for female mosquitoes to find oviposition sites (Siju et al., 2010), described in blood-fed females (Qiu et al., 2006b, 2013). Through antennal transcriptome analyses across mosquito species, dramatic changes in expression of chemosensory-related genes have been demonstrated in *An. gambiae* (Fox et al., 2001; Rinker et al., 2013a), *An. culicifacies* (De et al., 2018), *Ae. aegypti* (Matthews et al., 2016b), and *C. quinquefasciatus* (Taparia et al., 2017), and documented to occur from 24 h onwards following a blood meal, mirroring changes in ORN sensitivity and behaviour in female mosquitoes. For instance, *An. gambiae* blood-fed females display a general reduction of transcript abundance of chemosensory-related genes, coinciding with an overall decreased responsiveness to host-associated odours, observed beginning 12 h after a blood meal (Fox et al., 2001; Rinker et al., 2013a). Similarly, a gradual reduction in transcript abundance of almost all OBPs and ORs was observed in *An. culicifacies* within the first 30 min of blood feeding, followed by a recovery from 30 h to 72 h post-blood meal, which corresponds to the transition from host-seeking to oviposition behaviour of female mosquitoes (De et al., 2018). Interestingly, no significant changes in transcript abundance of these chemoreceptors were found following a second blood

meal, within the first gonotrophic cycle, suggesting a potential priming effect of the first blood meal exposure likely leading to a rapid host-seeking recovery in the consecutive gonotrophic cycles (De et al., 2018). Thus, behavioral transition from host-seeking to oviposition site selection observed in mosquitoes likely requires the modulation in sensitivity of particular chemosensory-related genes in response to ligands associated to both host and oviposition cues.

Changes in transcript abundance of chemosensory-related genes, previously shown to respond to host volatiles, illustrates that these receptors likely play a critical role in regulating the behavioural switch from host-seeking to oviposition site selection. For instance, ammonia and LA, both described as attractants for mosquitoes (Davis, 1984; Geier et al., 1999), were predicted to be ligands for OBP10 (Manoharan et al., 2013), which exhibits a suppression and rapid recovery in antennal transcript abundance in *An. culicifacies* blood-fed females (De et al., 2018). In *An. gambiae*, the down-regulation of OR1 transcript abundance following a blood meal (Fox et al., 2001), reflects a potential role of this receptor in host-seeking, since it was shown to respond to human-sweat components (Elissa A.Hallem, A.Nicole Fox, Laurence J.Zwiebel, 2004), and to phenol, which is required in the attraction of host-seeking female mosquitoes (Omondi et al., 2019). Similarly in female *C. quinquefasciatus*, OR1 is tuned to various host-associated volatiles (Xu et al., 2013), and is down-regulated following a blood meal (Taparia et al., 2017). Of note, the transcript abundance of the obligate co-receptors, ORco, IR8a, IR25a and IR76b, remain relatively unchanged following a blood meal in mosquitoes (Rinker et al., 2013a; Matthews et al., 2016b; Taparia et al., 2017), which suggests that the observed reduction in responsiveness to host-associated odours is likely due to the regulation of the other ORs. Changes in transcript abundance of genes involved in the detection of CO2 have been observed in the maxillary palps of female *Ae. aegypti* following a blood meal, as the neural sensitivity to this kairomone decreases in blood fed mosquitoes (Hill et al., 2019). In addition, the down-regulation of OR73 in the antennae of *C. quinquefasciatus* females, 24 h post blood meal, was proposed to be either linked to the suppression of host-seeking or a delayed onset of oviposition-associated behaviour (Taparia et al., 2017). Indeed, OR73 is sensitive to several phenolic compounds (Xu et al., 2013), which can be defined as both human-derived components (Elissa A.Hallem, A.Nicole Fox, Laurence J.Zwiebel, 2004; Curran et al.,

2005), and oviposition attractants (Allan and Kline, 1995b; Du and Millar, 1999).

Combined, these studies have shown that alterations in transcript abundance of chemosensory-related genes are correlated to the modulation of peripheral odour coding prior- and post-blood feeding, resulting in females undergoing a behavioural switch following blood feeding. As such, these studies represent a valuable resource for the identification of candidate receptors tentatively involved in the regulation of complex behavioural events following blood feeding. As the molecular mechanisms governing host-seeking suppression following a blood-meal are still poorly understood in mosquitoes, systematic efforts to deorphanize target receptors will be required to gain insights of the functional relevance of these observed changes in gene expression.

### 4.2.4 Changes in gene expression patterns upon pathogen infection

In mosquitoes, the infection by a pathogen has been demonstrated to induce modulation in the physiology and behaviour of the vector, which has been shown to be correlated with changes in transcript abundances (Sim et al., 2012; Gaburro et al., 2018)(Tallon et al. accepted). However, the mechanism(s) underlying such a behavioural modulation remain(s) largely unknown. Of note, transcriptomic analyses have shown that the modulation of gene expression profiles is quite similar between mosquitoes infected by different flaviviruses, *e.g.* West Nile virus, dengue virus, yellow fever, filarial worms and malaria (Bartholomay et al., 2010; Colpitts et al., 2011), suggesting a possible conservation of mechanisms upon infection. Transcriptome analyses of infected mosquitoes revealed an important number of canonical innate immune genes (Sanders et al., 2005; Bartholomay et al., 2010; Colpitts et al., 2011), and chemosensory-related genes (Adamo and Shoemaker, 2000; Abuin et al., 2011) to be differentially expressed upon infection. Changes in gene expression in the head of a mosquito, particularly increased abundance of calmodulin-dependent protein kinase transcripts, were shown to be associated with a loss of olfactory preference, and modulation in oviposition site selection of dengue-infected female *Ae. aegypti* mosquitoes (Gaburro et al., 2018). The silencing of two OBPs, OBP10 and OBP22, described to be enriched in dengue-infected female *Ae. aegypti*, resulted in an overall impaired efficiency of blood

acquisition, *i.e.* increased probing initiation time and reduced probing propensity (Sim et al., 2012). Thus, pathogen infection can lead to changes in expression patterns of genes which have been shown to be involved in chemosensation, suggesting dire implications in mosquito vectorial capacity.

Among the few chemosensory-related genes detected in the antennae and shown to be modulated by dengue infection, only the IR, *IR75k.1*, was less abundant in infected female *Ae. aegypti* (Tallon et al. accepted). Of the highly conserved mosquito antennal IRs, the orthologue complex IR75k in *An. gambiae* was found to be activated by carboxylic acids (Abuin et al., 2011; Jason Pitts et al., 2017), which are major components of human sweat involved in the regulation of host-seeking behaviour of most mosquito species (Cork and Park, 1996a; Qiu et al., 2006a; Carey et al., 2010). While *Plasmodium* parasites were previously shown to be able to alter odour profiles, including carboxylic acids, of infected hosts, resulting in the modulation of their attractiveness to the insect vectors (Shea et al., 2002; Moraes et al., 2014; Kelly et al., 2015; De Moraes et al., 2018), there is currently no evidence that dengue-infected human hosts produce different volatiles in comparison to healthy persons. The downregulation of *IR75k.1* observed in dengue-infected mosquitoes might contribute to a finer tuning of host selection, *i.e.* discrimination between healthy and infected hosts, and therefore deserves further investigation. Further analysis related to this IR, *e.g.* investigating changes in transcript abundance at several time point post-infection and with additional viral loads, should be considered. Furthermore, RNA-Seq of antennal transcripts revealed that the chemosensory-related and neuromodulatory gene families were likely not the main targets of the increased sensitivity to human odour described in dengue-infected female *Ae. aegypti* (Tallon *et al*. accepted). In contrasts, genes involved in neural signaling pathways might be involved in the enhanced host sensitivity of dengue-infected mosquitoes (Tallon *et al.* accepted), alike described in the case of neural-induced alterations in host attraction of female *An. gambiae* infected with Malaria (Emami et al., 2017). Moreover, similarly to that previously suggested (Matthews et al., 2016b; Latorre-Estivalis et al., 2017, 2020), it is interesting to note the possibility that genes involved in central functions, *i.e*. biogenic amines and neuropeptides, and encoding in neural circuits responsible for the processing of sensory stimuli, might inflect the sensitivity of neurons located in peripheral tissues, such as the antennae, through the recruitment of local regulatory pathways. Thus, changes in

transcript abundance of genes involved in neuromodulation and neural signaling pathways may guide disease vector behaviour, allowing infected mosquitoes to be more sensitive to host cues, and therefore transmitting pathogens more efficiently (Tallon *et al*. accepted).

The alteration of gene expression in response to infection was also shown to be both tissue- (Bartholomay et al., 2010; Colpitts et al., 2011; Bonizzoni et al., 2012; Shrinet et al., 2017), and time-specific (Sanders et al., 2005; Bartholomay et al., 2010; Colpitts et al., 2011; Bonizzoni et al., 2012; Choi et al., 2014; Raquin and Lambrechts, 2017). For instance, while infection generally results in a decrease in transcript abundance across these multiple tissues of infected mosquitoes (Colpitts et al., 2011; Bonizzoni et al., 2012; Shrinet et al., 2017), differentially regulated genes were generally fewer and mostly less abundant in the antennae (Tallon *et al.* accepted). The time-dependent gene expression patterns observed in pathogen-infected mosquitoes mirror viral temporal tropism, *e.g.* virus entry through the midgut, spread throughout the mosquito body, and reach of the salivary glands (Sanders et al., 2005; Bartholomay et al., 2010; Colpitts et al., 2011; Bonizzoni et al., 2012; Choi et al., 2014; Raquin and Lambrechts, 2017). Differential transcript accumulation, *i.e.* increase of number of genes between the first and the second week post-infection, was observed in the midgut and the salivary glands of *Ae. aegypti* infected by Dengue (Bonizzoni et al., 2012), as well as in the carcass, *i.e.* remainder of the body (Bartholomay et al., 2010). In contrasts, thoracic tissues of female *Ae. aegypti* mosquitoes, infected with filarial worms, were shown to undergo considerable changes in transcript abundance, particularly during the first two days following blood feeding (Choi et al., 2014). Overall, the dynamics of regulation of transcript abundances exhibit distinct temporal kinetics that likely reflect closely the histological phenotypes of infected mosquitoes (Brackney et al., 2008; Colpitts et al., 2011; Bonizzoni et al., 2012). For instance, proteins in the trypsin family previously demonstrated to enhance dengue infection in mosquitoes (Brackney et al., 2008), which were shown to be virus-down regulated in several tissues early in infection, were suggested to be involved in the innate immune response, while the gene named “matrix metallo-protease”, highly down-regulated in the salivary glands, late in infection, might be involved in controlling viral replication and transmission to a new host (Colpitts et al., 2011). In addition, virally-regulated gene expression was shown to be related to the levels of infection,

*e.i.* viral load detected in infected mosquitoes (Tallon et al. accepted). Among the differentially regulated functional classes, "oxidoreductase activity" and "transferase activity", all detected transcripts were more abundant in mosquitoes dengue-infected with the highest virus load (Tallon *et al.* accepted). In line with this study, oxidoreductive processes were previously shown to be highly upregulated in infected mosquitoes (Xi et al., 2008; Colpitts et al., 2011). It should be noted that the mechanism underlying the bimodal dengue-induced regulation of the physiology and behaviour between the first and second week post-infection, correlates to an antenna-specific increase in the abundance of predominantly neural signaling genes. Further expression analyses are necessary regarding specific transcripts identified as differentially abundant upon infection, as well as the identification of their key ligands, which inadvertently participate in enhancing insect vectorial capacity, therefore increasing the risk of disease transmission.

# 5. Summary of results

Female *Aedes aegypti* mosquitoes are the primary urban vector of several highly infectious human pathogens, including dengue fever, *i.e.* currently the fastest-growing infectious disease in the world. Disease transmission relies heavily on the ability of females to locate their human hosts. In mosquitoes, host-seeking is a behaviour primarily odour-mediated. The main goal of this thesis is obtain a better knowledge of the molecular parameters involved in the modulation of host-seeking in *Ae. aegypti*.

Following adult emergence, female mosquitoes gradually develop behavioural and physiological competence to host seek and blood feed, which generally take 2 to 3 days post-emergence, although females may have to wait longer before to take their first blood meal. Sugar seeking is therefore particularly essential for newly-emerged mosquitoes. Interestingly, both floral- and host-seeking inevitably linked to each other, yet fundamentally different. Moreover, males can be found in close proximity to humans, where they can find mates. Thus, host-seeking behaviour is dependent on both age and sex. In the first publication of this thesis, I have shown that the modulation of host-seeking and changes in the transcript abundance of the main chemoreceptor families, is both sex- and age-dependent in newly-emerged mosquitoes. The largest increase in transcript abundance described for select chemosensory genes, in the antennae between 1- and 3-days post-emergence, was found to reflect the increase in attraction to human odour observed in both males and females.

In *Ae. aegypti*, two ORs-of-interest, OR103 and OR4, have been shown in higher abundance in the human-preferring subspecies, *Ae. aegypti aegypti* compared to the zoophilic sub-species *Ae. aegypti formosus* (11). While OR4 respond to sulcatone, a compound present in high amounts in human odour (11), the function of OR103 remained to be determined. Moreover, changes in the abundance and coding regions of *OR4* have been found to alter the sensitivity of the encoded receptor to sulcatone, and suggested to be a major driver for the divergence of host selection between the two *Ae. aegypti* subspecies (11). In my second publication, OR103, together with OR117, which is the only OR to be more abundant in newly-emerged females compared to those displaying host-seeking (Tallon et al., 2019), have been selected to be heterologously expressed in the *Drosophila*

ab3A empty neuron system. These ORs were screened for responses to bioactive compounds in headspace volatile extracts collected from human body odour and the preferred host plant, *Lantana camara*, through combined gas chromatography and single sensillum recording analyses. Linalool, found in emanations from human body odour, was the only ligand for OR103. Interestingly, the allelic variation described in *OR103* affects the sensitivity of the encoded receptor to linalool, *.i.e.* the allele C which is inherited at a higher frequency in the zoophilic subspecies is more sensitive to linalool. In contrast, OR117 responded to two monoterpenes, camphor and eucalyptol, collected from *Lantana camara* floral extracts. The increased transcript abundance observed in OR117 in newly-emerged female mosquitoes, may therefore be part of a mechanism, *i.e.* a molecular switch, involved in the transition from floral- to host-seeking in female *Ae. aegypti* following adult emergence. Of note, both OR103 and OR117 belong the same Culicinae-specific OR clade.

Vector-borne pathogens have the capacity to manipulate their hosts by affecting survival, reproductive performance, and sugar-seeking, as well as probing and biting activities, thereby impacting the cycle of disease transmission. In the third publication of this thesis, the modulation of locomotion and host seeking in female *Ae. aegypti* by dengue infection is shown to be time-dependent, *i.e.* females increase locomotor activity at 4-6 days post-infection, but do not alter their odor-driven host-seeking response, while females are less active at 14-16 days post-infection, yet more sensitive to human odours. This observed increased in physiological and behavioural sensitivity was shown to coincide with changes in the expression patterns of genes involved in neural signalling pathways, expressed in the antennae of females at 14 days post-infection. Thus, the sensitivity of the mosquito peripheral olfactory system appears to be altered by dengue infection by enhancing the overall neural responsiveness of the antenna, rather than the selective regulation of chemosensory-related genes. While dengue virus enhances vector-related behaviors, i.e. decreases risks of predation and increases spatial exploration, during the early stages of infection, the virus enhances the host-seeking capacity of the vector likely resulting in an increased risk of virus transmission at the later stages post-infection.

In summary, this thesis may represent an essential step towards understanding better the modulation of host-seeking (publication I), and the transition from floral- to host-seeking in newly-emerged mosquitoes

(publication II), as well as the biting specialisation on humans in *Ae*. *aegypti* (publication II), and the modulation of host-seeking in dengue-infected mosquitoes (publication III).

# 6. Conclusion and perspectives

In mosquitoes, sugar and host-seeking, rely heavily on olfaction. The modulation of these behaviours is correlated with changes in the expression patterns of various chemosensory-related gene families, depending on multiple factors, including the tissue considered, sex, age, physiological state, and infection by a pathogen. In my first publication, newly emerged mosquitoes was shown to coincide with changes in transcript abundance of the main chemoreceptor families, in both a sex- and age-dependent manner. Of particular interest is the decrease in transcript abundance of one particular receptor, OR117, which correlates with the observed increased attraction to human odours as the mosquito matures.

Even though it is well-established that vector-borne pathogens have the capacity to manipulate their hosts, and influence their cycle of disease transmission, the molecular mechanisms underlying the behavioural modulation caused by the infection remains unclear. In my second publication, the modulation of locomotion and host-seeking in female *Ae. aegypti* following dengue infection is shown to be time-dependent, and coincide with changes in transcript abundance of genes involved in neural signaling pathways. Although the mechanism underlying this change in behavior remains unknown, this study represents an essential step towards understanding better the modulation of host-seeking in infected mosquitoes. Moreover, the increased mobility and local spread of infected mosquitoes, together with the enhanced propensity to host seek, which may increase the risk of multiple blood meals from multiple hosts, intensifies the interaction between infective mosquitoes and humans, inadvertently increasing the risk of dengue transmission.

In recent years, the efficient identification of candidate behaviour-modulating volatiles, and the emergence of techniques to express ORs in heterologous systems have permitted researchers to reliably identify key ligands for these receptors (Elissa A.Hallem, A.Nicole Fox, Laurence J.Zwiebel, 2004; Lu et al., 2007; Bohbot and Dickens, 2009; Bohbot et al., 2011a, 2013a; McBride et al., 2014; Omondi et al., 2015c). The functional characterisation of OR117 and OR103, previously hypothesised to be involved in host discrimination in *Ae. aegypti*, adds important information to our understanding of the functional role of individual members in the large repertoire of olfactory receptors. Even though scientists have acquired an

increased knowledge of insect olfaction over the past decades, the identity of bioactive compounds and the molecular mechanisms underlying the modulation of odour-mediated behaviour remain poorly understood in mosquitoes.

To further assess the role of the ORs identified and analysed functionally in this study, ongoing behavioural analysis of CRISPR-Cas9, *OR103* and *OR117* knock out mutant *Ae. aegypti* are currently being performed. Moreover, a systematic functional analysis of the near complete repertoire of *Ae. aegypti* ORs is being conducted using ecologically relevant compounds in order to define the odour space of female *Ae. aegypti*. This study has identified amino acid residues that are likely involved in regulating ligand binding. Future studies should be aimed at identifying which of these constituents are involved in the modulation of OR sensitivity and specificity through mutations of single or multiple amino acids. The recent advances of fast-developing genetic tools (Tian et al., 2009; Hammond et al., 2016; Dong et al., 2018; Knott and Doudna, 2018; Riffell, 2019) have allowed a more detailed research on the molecular machinery underlying the modulation of olfactory responses in insects. Overall, these tools are likely to contribute to a more comprehensive and integrated understanding of the molecular basis of host-seeking modulation in mosquitoes.

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# Popular science summary

Le moustique *Aedes aegypti* est le principal vecteur urbain de transmission de nombreuses maladies infectieuses et tropicales, dont la dengue, la maladie qui se propage actuellement le plus rapidement au monde. La transmission d'une maladie infectieuse se repose fortement sur des comportements liés à l'olfaction, comme par exemple la recherche de sucre et d'hôte. Dans la mesure où les hydrates de carbone d'origine végétale sont une source d'énergie pour les activités quotidiennes, les aliments sucrés fournissent de l'énergie pour les fonctions somatiques, telles que le vol, la survie et la reproduction, et ont par conséquent un impact considérable sur la capacité vectorielle des moustiques. De même, la recherche d'hôte, qui dépend de la capacité des femelles à localiser avec succès et à se nourrir de sang sur un hôte humain, est intimement liée à la transmission de maladies infectieuses. De plus, la recherche d'hôte est une étape cruciale dans le cycle de vie de la femelle, l'alimentation en sang étant indispensable pour compléter le développement ovarien. Il est intéressant de noter que les mâles réagissent et s'orientent également en direction des signaux émis par les hôtes, en particulier durant l'accouplement. Une fois l'émergence accomplie, les femelles développent les compétences physiologiques et comportementales nécessaires à la recherche d'hôte en l'espace de deux à cinq jours, et peuvent avoir à attendre davantage avant de se nourrir de sang pour la première fois. La recherche de sucre est donc particulièrement vitale pour les moustiques nouvellement émergés. La recherche d'hôte et de sucre sont des comportements profondément différents mais intimement liés. En effet, la disponibilité en sucre de l'environnement influe sur la probabilité de recherche d'hôtes humains et la fréquence de multiples repas sanguins, et a par conséquent des implications épidémiologiques considérables. Par ailleurs, les agents pathogènes à transmission vectorielle, tel que le virus de la dengue, ont la faculté d'altérer la recherche d'hôte chez le moustique, et peuvent alors augmenter le risque de transmission de maladies. La recherche d'hôte chez le moustique dépend donc de facteurs multiples, comme l'âge,

le sexe, l'état physiologique et l'infection par un pathogène. Chacun de ces facteurs est exploré plus en détail dans cette thèse.

L'olfaction est l'un des systèmes sensoriels les plus sophistiqués chez les insectes, et le principal sens utilisé pour la recherche et localisation des hôtes végétaux et animaux. Dans la nature, les insectes doivent discriminer les substances odorantes volatiles d'intérêt biologique pour eux, et ceci parmi une multitude de composés odorants volatiles émis à différentes proportions par des sources hôtes et non-hôtes. Chez le moustique, les organes olfactifs (les antennes, les pièces buccales, les palpes maxillaires et la trompe) sont recouverts d'une structure sensorielle poreuse, la sensille, qui abrite les neurones récepteurs olfactifs. Le fondement même du système olfactif du moustique repose sur la régulation de l'expression génique de nombreux chimiorécepteurs exprimés dans ces neurones récepteurs olfactifs. La première publication issue de cette thèse démontre que les modulations de recherche d'hôte chez les moustiques nouvellement émergés coïncident avec des changements d'expression de ces gènes (*e.g.* diminution du nombre de copies transcrites du récepteur odorant OR117 lorsque les moustiques deviennent capables de rechercher un hôte). Par conséquent, après avoir caractérisé fonctionnellement OR117, conjointement avec un autre récepteur odorant OR103, j'ai pu démontrer que ces deux récepteurs répondent aux monoterpènes. Le rôle potentiel de ces récepteurs dans la transition entre la recherche de sucre vers la recherche ou l'évitement de l'hôte est discuté plus en détail dans la troisième publication issue de cette thèse. Enfin, la seconde publication met en évidence la modulation de recherche d'hôte chez la femelle *Ae. aegypti* lors d'une infection au virus de la dengue, en coïncidence avec des changements dans le nombre de copies de gènes impliqués dans les voies de signalisation neuronales, et exprimés dans les antennes des femelles. Avec l'essor du séquençage nouvelle génération, des systèmes hétérologues d'expression génétique et de la disponibilité grandissante de génomes de différents moustiques, cette thèse représente une contribution majeure aux connaissances sur l'olfaction du moustique. Comprendre le fonctionnement des comportements influencés par l'olfaction est crucial pour le développement de stratégies de lutte efficaces face aux vecteurs de maladies infectieuses et tropicales.

# Populärvetenskaplig sammanfattning

*Aedes aegypti* myggan är den primära vektorn för olika infektiösa tropiska sjukdomar. Sjukdomsspridning är beroende av doftstyrda beteenden t.ex. värdsökande, som är avgörande under myggans livscykel och har stor inverkan på vektorkapaciteten. Doftsinnet är ett av de mest sofistikerade sensoriska systemen och med hjälp av det, lokaliserar och diskriminerar insekter biologiskt relevanta doftämnen. Honornas värdsökande observeras i allmänhet inte förrän 2-3 dagar efter de har nått vuxen ålder.

Hanarna har också visat sig svara på och orientera sig mot värdassocierade doftsignaler. Vektorburna patogener kan förändra värdens beteende och fysiologi och därmed öka risken för överföring av sjukdomar. Således beror moduleringen av värdsökandet på flera faktorer t.ex. ålder, kön och infektion med en patogen, som är huvudfokus för denna avhandling. Under mitt doktorandprojekt har jag visat att moduleringen av värdsökandets början sammanfaller med förändringar i transkriptionsflod av kemosensorierelaterade gener uttryckta i antennerna för nyfödda myggor. Av dessa gener uppvisar en speciell doftreceptor, OR117, en minskning av transkriptionsöverskott när honorna blir kapabla att söka värd. Senare har jag funktionellt karaktäriserat OR117, tillsammans med en annan intressant doftreceptor, OR103, som tidigare har visat sig vara mer riklig i de underarter av *Ae. Aegypti* som föredrar människor som värdar. Både OR117 och OR03 är anpassade till monoterpener, och deras potentiella roll i övergången mellan socker- till värd-sökande respektive mänskligt undvikande diskuteras. Vidare har jag visat att rörelsen och värdsökandet moduleras hos dengue-infekterade honor på ett tidsberoende sätt, dvs beroende på tiden efter infektionen, och sammanfaller med förändringar i transkriptionsflod av gener involverade i neurala signalvägar. En bättre förståelse för modulering av doftstyrda beteenden hos myggor är avgörande för utvecklingen av effektiva kontrollstrategier mot sjukdomsvektorkontroll.

# Acknowledgements

The long journey of a PhD is never fully safe, even less foreseeable … this is why you cannot do it on your own.

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For all, I would have forgotten… you are on the last page ;)

And for you **reader**, if you made it up to here… Here is 10 "crazy" facts that I came across during the writing of this thesis :

1. Despite their rather charming name, mosquitoes are the world's deadliest animals, when considered the number of human life claimed per year [1,2]
2. During a blood meal he average amount of blood taken by *Ae. aegypti* is 4,2 µl at the rate of uptable of ca. 0,0016 µl $s^{-1}$ [3]
3. After locating and biting a host, female mosquitoes become engorged on a blood-meal that can exceed their unfed body weight.
4. The Roman poet and philosopher, Titus Lucretiu Carus, has already emitted the hypothesis of the existence of a large variety of odours because each odorant possesses an unique structure (50 BC) [4]
5. While the name of the disease "yellow fever" comes from the probability of infected patients to develop jaundice (yellowing of the skin and eyes when the liver and the kidneys become infected) [5], the name "Chikungunya" comes from a Makonde word describing the bent posture of persons with the severe arthralgia, which is the hallmark of chikungunya fever, the disease caused by the virus [6]
6. While at the beginning of the 20th century, dengue outbreaks were common in the Mediterranean basin, the last major epidemic on the European continent occurred in 1927/28 in Greece. Some time after this severe outbreak, leading to more than a thousand deaths, both dengue and its primary vector, *Ae. aegypti*, 'abandoned' the continent [7]
7. Once infectious, the mosquito is capable of transmitting the virus of dengue for the rest of its life [8]

8. In the shadow in the coronavirus, we tend to forget the progress of dengue virus worldwide, only six months following the upgrade by of WHO of dengue among the top 10 of worst diseases in the world (World Health Organization, 2019), an increase of 500% of dengue cases were reported in Brazil only [9]
9. While flying, mosquitoes are capable of detecting upwind hosts, *i.e.* fluctuations in $CO_2$ concentrations above ambient ($0.035% or 350ppm) over distances of up to 10 meters [10]
10. In the field of olfactory psychophysics, *i.e.* the study of the odour perception, the number of olfactory stimuli which can be detected and discriminated by humans is estimated to be 10,000 discrete odors, although it has also been suggested to reach up to the astonishing number of 1 trillion [11]. Comparably very little is known in insects. Moreover, the existing number of odorants emitted by any livings on Earth remains unknown.

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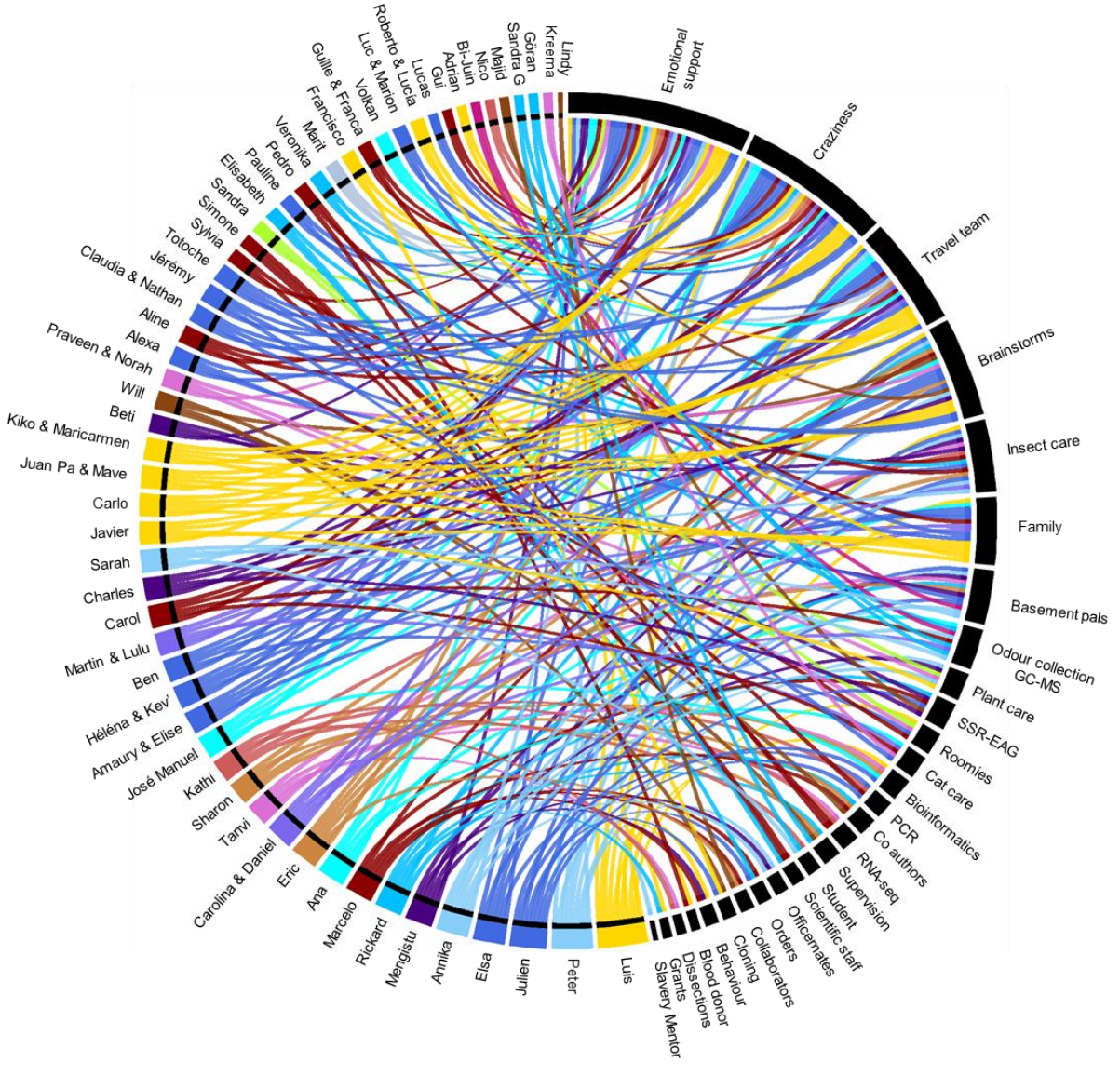
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Host seeking, which is primarily odour-mediated, is a crucial step of mosquito life cycle, and a key determinant of their vectorial capacity. The modulation of host seeking relies on several factors, e.g. sex, age, infection by a pathogen, and reflects changes in the expression of chemosensory-related genes. Transcriptome analysis and functional characterisation of these chemoreceptors have provided a good insight into novel control strategies against disease vectors.

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